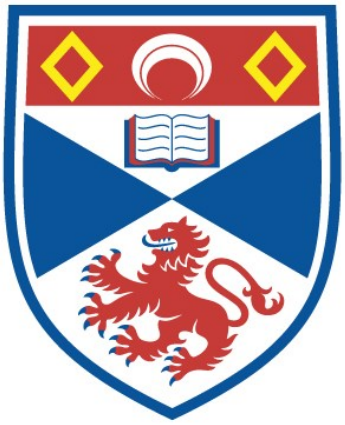
THE MOLECULAR BASIS OF THE ACCLIMATIZATION OF PLANTS TO CHILLING TEMPERATURES

John Wilson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1974

THE MOLECULAR BASIS OF THE ACCLIMATIZATION OF PLANTS TO CHILLING TEMPERATURES

JOHN WILSON

A thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews, July 1974.

The Molecular Basis of the Acclimatization of Plants to Chilling Temperatures

John M. Wilson

(A thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews, July, 1974)

ABSTRACT

Leaves of tropical and sub-tropical species are rapidly chill-injured when exposed to temperatures in the 0 to 10°C range. In contrast, leaves of temperate and arctic alpine species can withstand these temperatures without damage. Chill-sensitive leaves can be divided into two categories based on their susceptibility to chilling-injury:-

1) Extremely chill-sensitive species which are rapidly damaged on exposure to temperatures between 12 to 15°C (e.g. Episcia reptans) and which cannot be readily hardened against chilling-injury. Maintaining 100 per cent relative humidity during chilling at 5°C does not prevent injury to these species, although the speed at which injury occurs is reduced.

2) Chill-sensitive species which are damaged in the 0 to 10°C range (e.g. Phaseolus vulgaris) and which can be readily hardened against chilling-injury at 5°C, 85 per cent RH, by 4 days growth at 12°C, 95 per cent RH, before chilling. Maintaining 100 per cent RH during chilling at 5°C prevents injury to these species.

It is considered that the primary response in chilling-injury is a temperature induced phase transition in the lipids of the cellular membranes

from a liquid-crystalline to a solid gel state. The temperature at which the phase change occurs appears to be determined mainly by the degree of unsaturation of the fatty-acids associated with the phospholipids. The chill-sensitivity of leaves grown at 25°C was related to a low percentage of linoleic and linolenic acid associated with each phospholipid. The degree of unsaturation of the glycolipids could not be related to the chill-sensitivity of the species.

Hardening the chill-sensitive species Phaseolus vulgaris and Gossypium hirsutum against chilling-injury at 5°C, 85 per cent RH, resulted in increases of up to 12 per cent in the percentage of linoleic acid associated with all the leaf phospholipids. The degree of unsaturation of the glycolipids did not change during hardening. The increases in unsaturation of the phospholipids were shown to be positively related to the increased tolerance of the plants to chilling by the fact that similar increases did not occur during the growth of chill-resistant Hordeum vulgare at 12°C and the ineffective attempts at hardening chill-sensitive Episcia reptans over 4 days at 15°C, the lowest temperature this species can withstand without injury.

Chilling-injury in Phaseolus vulgaris could be prevented by enclosing the plants in polythene bags at 5°C, thus maintaining 100 per cent RH. However, leaves of this species transferred from 25°C to 5°C, 100 per cent RH and 12°C, 100 per cent RH for 4 days did not harden against subsequent chilling-injury at 5°C, 85 per cent RH. In agreement with this finding no increase in unsaturation of the phospholipids was detected over 4 days growth at 5°C, 100 per cent RH, and 12°C, 100 per cent RH.

It is suggested that plants grown at 5 and 12°C, 100 per cent RH, by enclosure in polythene bags do not harden against subsequent chilling-injury at 5°C, 85 per cent RH, because the carbon dioxide concentration within the bag is rapidly lowered by photosynthetic fixation. This results in the cessation of photosynthesis and a reduced supply of the cofactors oxygen and NADPH available for desaturase activity, thereby preventing an increase in the degree of unsaturation of the phospholipids.

An increase in leaf age at 25°C was shown to increase the susceptibility of chill-sensitive plants to chilling-injury. This increase in susceptibility of older leaves to damage was related to a decrease in the degree of unsaturation and weight of phospholipids with increase in physiological age at 25°C.

In conclusion, the results reported in this investigation provide evidence that hardening chill-sensitive leaves prevents chilling-injury by increasing the degree of unsaturation of the membrane phospholipids thereby lowering the transition temperature of the lipid layer of the cellular membranes. This phase change does not occur on chilling chill-resistant plants but may occur at sub-zero temperatures and increase their susceptibility to freezing-injury.

CAREER

I entered the University of Liverpool in October, 1967 and graduated in July, 1970 with an upper second class Honours degree in Botany.

From October, 1970 to September, 1971 I studied for the degree of M. Sc. in Ecology at Aberdeen University and graduated in October, 1971.

In October, 1971 I matriculated at the University of St. Andrews under Ordinance General No. 12 and later as a candidate for the degree of Ph. D. under Resolution of the University Court, 1967. No. 1.

In May 1974 I was awarded an Ernest Cook Trust Research Fellowship by the Royal Society for 2 years from October 1st, 1974 to enable me to continue with my research on the chill-sensitivity of tropical plants at the Botany Department, University of St. Andrews.

DECLARATION

I hereby declare that this thesis is based upon work done by myself, that the thesis is my own composition, and that it has not been previously presented for a higher degree.

The research work was carried out in the Department of Botany, University of St. Andrews, under the supervision of Dr. R. M. M. Crawford.

CERTIFICATE

I hereby certify that John Wilson has been engaged upon research work for a minimum of nine terms under my supervision, that he has fulfilled the conditions of Ordinance No. 12, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

I wish to express my appreciation to my supervisor, Dr. R. M. M. Crawford for his helpful advice and enthusiasm over the past three years. In particular, I wish to thank my wife, Frances Wilson, for typing the thesis and her encouragement during the last three years.

I also wish to thank Professor J. A. Macdonald, in whose department the work was carried out, and Professor F. D. Gunstone for his expert assistance with the separation and identification of leaf fatty-acids.

Finally, I wish to thank the staff of the Botanic Garden for their invaluable help in growing the large numbers of plants used in this investigation, and the staff of the Botany Department.

From October, 1971 to September, 1973 the research was carried out during the tenure of an N. E. R. C. Research Studentship. I am very grateful to have been awarded a St. Andrews University Research Scholarship for the final year of my research from October, 1973 to October, 1974.

CONTENTS

PAGE

INTRODUCTION 1

CHAPTER ONE : THE PLANT MATERIALS

1. SUSCEPTIBILITY TO CHILLING-INJURY 18
2. THE PLANT SPECIES 21
3. GROWTH CONDITIONS 24
4. SYMPTOMS OF CHILLING-INJURY 25
5. HARDENING IN THE LIGHT 28
6. HARDENING IN THE DARK 32
7. THE EFFECTS OF 100 PER CENT RELATIVE HUMIDITY ON CHILLING-INJURY AND HARDENING 35
8. ESTIMATION OF CHILLING-INJURY 37

A) The effectiveness of hardening at 12°C, 95 per cent RH 38

B) The effect of leaf age on the susceptibility to chilling-injury 40

C) Variation in the susceptibility of individual leaves to chilling-injury 42

D) The effect of light intensity on the severity of chilling-injury 45

9. THE EFFECTIVENESS OF THE HARDENING TREATMENT AGAINST PROLONGED CHILLING AT 5°C, 85 PER CENT RH 46

CHAPTER TWO : THE METABOLIC CONSEQUENCES OF CHILLING-DAMAGE TO THE MITOCHONDRIAL MEMBRANES OF CHILL-SENSITIVE PLANTS

INTRODUCTION 47

MATERIALS AND METHODS

1. Growth conditions 50
2. Respiratory measurements 50
3. Ethanol concentration in the leaves 51

PAGE

RESULTS

1. THE EFFECT OF CHILLING ON THE RESPIRATION RATE OF CHILLED LEAVES ON RETURN TO 25°C 53
2. THE EFFECT OF CHILLING ON THE RATES OF GLYCOLYTIC AND TRICARBOXYLIC ACID CYCLE ACTIVITY

A) The differential effect of malonate on the respiration of leaves before and after chilling 56

B) Ethanol accumulation in chilled chill-sensitive plants 58

DISCUSSION 60

SUMMARY 63

CHAPTER THREE : LEAF AND CHLOROPLAST FATTY-ACID CONTENT IN RELATION TO HARDENING AND CHILLING-INJURY

INTRODUCTION 64

MATERIALS AND METHODS

1. Growth and hardening conditions 68
2. Leaf material and chloroplast isolation 68
3. Lipid extraction 69
4. Preparation of the methyl esters of the fatty-acids 69
5. Gas-liquid chromatography 70

RESULTS

1. THE IDENTIFICATION OF THE METHYL ESTERS OF THE LEAF FATTY-ACIDS 71
2. A COMPARISON OF THE LEAF AND CHLOROPLAST FATTY-ACID COMPOSITION OF CHILL-SENSITIVE AND CHILL-RESISTANT PLANTS GROWN AT 25°C 75
3. CHANGES IN THE LEAF AND CHLOROPLAST FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON HARDENING AT 12°C, 95 PER CENT RH 75

4. CHANGES IN THE LEAF FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON CHILLING AT 5°C, 85 PER CENT RH 80
5. CHANGES IN THE CHLOROPLAST FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON CHILLING AT 5°C, 85 PER CENT RH 85
6. CHANGES IN THE LEAF FATTY-ACID COMPOSITIONS OF HARDENED LEAVES DURING CHILLING AT 5°C, 85 PER CENT RH 85

DISCUSSION 90

SUMMARY 95

CHAPTER FOUR : THE ACCLIMATIZATION OF PLANTS TO CHILLING TEMPERATURES IN RELATION TO THE FATTY-ACID COMPOSITION OF LEAF POLAR LIPIDS

INTRODUCTION 96

MATERIALS AND METHODS

1. Growth and hardening conditions 101
2. Lipid extraction 103
3. Thin-layer chromatography 103
4. Identification of lipids 104
5. Preparation of the methyl esters of the fatty-acids 105
6. Gas-liquid chromatography 107

RESULTS

1. A COMPARISON OF THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE AND CHILL-RESISTANT LEAVES GROWN AT 25°C 108
2. THE EFFECTS OF HARDENING

A) The effect of hardening and leaf age on the susceptibility of chill-resistant Hordeum vulgare to freezing-injury at -4°C 128

B) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive and chill-resistant species on hardening at 12°C, 95 per cent RH 130

C) Changes in the weights of the polar lipids of chill-sensitive and chill-resistant species on hardening at 12°C, 95 per cent RH 139

3. THE EFFECTS OF CHILLING AT 5°C, 85 PER CENT RH

A) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive and chill-resistant species on chilling at 5°C, 85 per cent RH 143

B) Changes in the weights of the polar lipids of chill-sensitive and chill-resistant species on chilling at 5°C, 85 per cent RH 154

4. THE EFFECTS OF CHILLING AT 5°C, 100 PER CENT RH AND GROWTH AT 12°C, 100 PER CENT RH

A) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive Phaseolus vulgaris on chilling at 5°C, 100 per cent RH and growth at 12°C 100 per cent RH. 157

B) Changes in the weights of the polar lipids of chill-sensitive Phaseolus vulgaris on chilling at 5°C, 100 per cent RH and growth at 12°C, 100 per cent RH 158

DISCUSSION

1. A COMPARISON OF THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE AND CHILL-RESISTANT LEAVES GROWN AT 25°C 168

2. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES DURING HARDENING AT 12°C, 95 PER CENT RH 169

3. CHANGES IN THE FATTY-ACID COMPOSITIONS AND WEIGHTS OF THE POLAR LIPIDS OF CHILL-RESISTANT HORDEUM VULGARE DURING HARDENING AT 5 AND 12°C AGAINST FREEZING-INJURY AT -4°C 173

PAGE

4. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES ON CHILLING AT 5°C, 85 PER CENT RH 175
5. CHANGES IN THE WEIGHTS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES ON CHILLING AT 5°C, 85 PER CENT RH 177
6. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE PHASEOLUS VULGARIS ON CHILLING AT 5°C, 100 PER CENT RH AND GROWTH AT 12°C, 100 PER CENT RH 179
7. CHANGES IN THE FATTY-ACID COMPOSITIONS AND WEIGHTS OF THE POLAR LIPIDS DURING THE GROWTH OF CHILL-SENSITIVE AND CHILL-RESISTANT PLANTS OVER 4 DAYS AT 25°C 182

SUMMARY 184

FINAL DISCUSSION 187

FINAL SUMMARY 201

BIBLIOGRAPHY 208

LIST OF ABBREVIATIONS

Fatty-acids	Common name
*12:0	Lauric acid
14:0	Myristic acid
16:0	Palmitic acid
16:1	-
16:2	-
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	Linolenic acid

Lipids

Glycolipids

MGDG	Monogalactosyl diglyceride
DGDG	Digalactosyl diglyceride
SL	Sulpholipid

Phospholipids

PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
PA	Phosphatidic acid
PG	Phosphatidyl glycerol

Others

ATP	adenosine triphosphate
cms	centimetres
cv	cultivar
D	denatured state of protein

*Ratio shown denotes the number of carbon atoms to the number of double bonds in the molecule

E_a	activation energy
g	gram
G	force due to gravity
G. L. C.	gas-liquid chromatography
H_g	mercury
m	metre
M	molar
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
N	native state of protein
NADPH	nicotinamide dinucleotide phosphate (reduced)
P	page
RH	relative humidity
RQ	respiratory quotient
SH	sulphydryl group of proteins
sq	square
SS	covalent disulphide bond of proteins
TCA	tri-carboxylic acid cycle
μg	microgram
μl	microlitre
μm	microns
W. M. $^{-2}$	watts per square metre
$\overline{X}$	mean value

INTRODUCTION

The effect of low temperature on leaves is probably the most important ecological factor limiting the survival of many species, both wild and cultivated at the polar limits of their distribution. Although it has been known since 1778 (Bierkander) that the leaves of tropical and sub-tropical species are rapidly chill-injured when exposed to temperatures in the chilling range (0-10°C), the cause of injury is still not clear. Early workers noted that animals (Pearson and Raper, 1927) and the seed oils of plants (Hilditch, 1956) originating from cold climates had more unsaturated fatty-acids in their lipids and it was suggested that death of tropical species at low temperatures could be due to solidification of the cellular lipids. Therefore, most studies on chilling-injury have been centered on the fatty-acid composition and physiological activity of membrane preparations. Because relatively pure plant mitochondria can be prepared from fruits and storage organs without chloroplast contamination, and due to the economic importance of being able to extend the storage life of chill-sensitive fruits at low temperatures, it is not surprising that the majority of experiments on fatty-acid composition and chilling-injury have been carried out with fruit and vegetable preparations.

There are, however, dangers in extrapolating the effects obtained with fruit and vegetable mitochondria to account for the behaviour of leaves in relation to chilling-injury. Leaves of the majority of chill-sensitive species are rapidly chill-injured and show signs of necrosis

after only a few hours at the chilling temperature (P. 27). In contrast, the first visible symptoms of chilling-injury in fruits usually take several days to develop. During the first hours of chilling leaves suffer very rapid water loss (P. 27) and leakage of electrolytes which suggests that the first detrimental changes may take place in the plasmallema rather than the mitochondria. Furthermore, leaves contain a far higher proportion of chloroplasts than fruits and detrimental changes associated with these and other membranes may occur before that of the mitochondria. Perhaps the most important difference between leaves and fruits is that leaves can be progressively hardened to withstand low temperatures either in the chilling range or in the sub-zero frost damage range. The extremes of temperature which most organisms can withstand depends largely on the environmental conditions to which they have been previously exposed. The extension of these extremes by either heat or cold exposure to an intermediate temperature is called hardening. The present study concerns the physiological changes that occur in response to a decrease in temperature.

Because attempts at hardening chill-sensitive fruits met with little success this led to the view that it is not possible to harden chill-sensitive leaves to any significant degree (Lyons, 1973). However, hardening of chill-sensitive cotton seedlings (Stewart and Guinn, 1969) and tomato seedlings (Wheaton and Morris, 1967) was found to require 2 days at a temperature slightly above the chilling range for maximum protection against chilling-injury. The present author has found that a longer period of 4 days at 12^{o}C was necessary

for the maximum protection of older plants of Gossypium hirsutum, Phaseolus vulgaris and Cucumis sativus against chilling-injury at 5°C, 85 per cent RH. It is of particular interest that the leaves of some chill-sensitive species do not harden with the same facility as the above species. Episcia reptans fails to harden at 12°C over a 4-day period and is severely injured when exposed to this temperature for 48 hours. Furthermore, hardening of this species at 15°C over 4 days provides no protection against chilling at 8°C. Species of this type may require several months at the hardening temperature to afford protection against chilling-injury. Plants of Episcia reptans had to be kept in a cool greenhouse (15-20°C) for 3 months in order to be able to withstand chilling at 12°C for 4 days. In contrast, the majority of fruits and vegetables cannot be readily hardened even by prolonged periods of acclimatization possibly because they are mature organs and unable to respond with the same plasticity as the growing leaf. Attempts at hardening sweet potatoes have not been successful in reducing chilling-injury (Wheaton and Morris, 1967) and hardening of cucumbers and bananas was only effective against slight chilling (Apeland, 1966; Pantastico, Grierson and Soule, 1967). Thus, any extrapolation of observations on mitochondria of fruits and vegetables to account for either the mechanisms of damage or resistance to low temperatures by whole plants neglects three essential facts:-

1) in the leaf chilling-injury occurs at a much faster rate than in fruits;
2) in the leaf the first detrimental changes may occur in the plasmallema and chloroplasts rather than the mitochondria;

3) leaves differ from fruits in that they can be hardened to withstand low temperatures.

Therefore in studying the role of unsaturated fatty-acids in the acclimatization of plants to low temperatures it is essential to construct experiments which examine the changes that take place in the leaf membranes during hardening and compare these changes with the effects produced in plants in which no hardening is achieved. Only in this way is it possible to determine if the changes in unsaturation in those species which harden are related to the requirement for a specific physical state of the lipid layer of cell membranes or whether the changes are simply the indirect result of temperature on the biosynthetic mechanism, unassociated with the development of chill-resistance.

Lipid Studies

The term 'lipid' is often used to denote a wide variety of natural products including fatty-acids, steroids, terpenes, and carotenoids, which have in common a ready solubility in organic solvents such as ether, hexane, benzene, chloroform or methanol. However, a more specific term is to be preferred and in this thesis the term 'lipid' is restricted to fatty-acids and their derivatives or metabolites. A classification of leaf lipids into either neutral or polar lipids is shown in Table 3 .

Research on the physical properties of the membranes of chill-sensitive plants and homeothermic animals at chilling temperatures by spin labelling has shown that a phase change occurs in the lipid layer of the membranes from a liquid-crystalline to a solid gel

state (Raison, 1973). The two most important effects of a phase change in the membrane lipids of the leaves of chill-sensitive species on chilling are an increase in membrane permeability and an increase in E_a of membrane bound enzymes. Increased membrane permeability may occur on chilling due to the solidification of the membrane lipids and their contraction, thus causing 'cracks' or 'holes' to appear in the membrane. Below 4°C these cracks may be accentuated by the expansion of water. In addition, Träuble and Haynes (1971) have suggested, from studies of artificial lipid-water systems, that increased membrane permeability accompanies the phase transition due to:-

a) A decrease in membrane thickness.

b) Changes in the structure of the hydrocarbon chains which are important for diffusion across the membrane.

c) Changes in the arrangement of the polar head groups which are important for the process of entry of the permeants into the membrane.

An increase in the permeability of the plasmallema and tonoplast may lead to the death of the leaf by dehydration and an increase in E_a of enzymes may lead to injury by producing metabolic imbalances. However, if no degenerative injury occurs during chilling, the phase change is completely and instantaneously reversible on return of the plants to the warmth. Similar changes are not found in poikilothermic animals and chill-resistant plants, organisms which can function down to or below 0°C. The major factors which are thought to determine the temperature of the phase change are shown in Table 1. The degree of unsaturation of the fatty-acids is the most important of these factors, as shown by the increase in unsaturation of the fatty-acids during acclimatization of

Table 1. An outline of the factors which may determine the temperature at which the phase change occurs in living membranes

Factors	References
1) The degree of unsaturation of the fatty-acids. The higher the degree of unsaturation the lower the temperature at which the phase change occurs.	Lyons and Asmundson, 1965.
2) The length of the fatty-acid chain. In general the shorter the fatty-acid chain the lower the transition temperature.	Ladbrooke and Chapman, 1969.
3) The configuration of the fatty-acids. The transition temperature of the cis fatty-acids is lower than trans fatty-acids.	Gunstone, 1967.
4) The inclusion of cholesterol or other 'cryoprotective' agents in the membranes. An increase in the level of cholesterol lowers the transition temperature.	Steim, Tourtellotte, Reinert, McElhaney and Rader, 1969.
5) The variation in the amount of a particular lipid component in the membrane.	
6) The variation of the pH in the region of the membrane. The lower the pH, the lower the transition temperature.	Träuble, 1972.
7) The water content of the lipid. An increase in hydration lowers the temperature of the phase change.	Ladbrooke and Chapman, 1969.

Table 1. Continued...

Factors	References
8) Interaction between different phospholipids. Mixing of phospholipids may lower the temperature of the phase change below that of any of the component lipids of the mixture - as indicated by the increased permeability of the lipid mixture.	De Gier, Mandersloot and Van Deenen, 1968.
9) The interaction of structural proteins and lipids may affect the fluidity of the lipid hydrocarbon chains.	Chapman, Kamat, De Gier and Penkett, 1968.
10) Various minor factors such as the double bond position in unsaturated fatty-acids.	Gunstone, 1967.

plants as well as poikilothermic and homeothermic animals to low temperatures (Table 2). It is thought that an increase in unsaturation during hardening may prevent chilling-injury in plants by lowering the temperature at which the phase change occurs in the lipid layer of the cellular membranes.

Most investigations on the changes in fatty-acid composition during hardening to sub-zero temperatures have been made on non-photosynthetic tissues (Table 2) and no investigations have been made on the fatty-acid composition of leaf membranes in relation to hardening against chilling-injury. Analyses of non-photosynthetic tissues such as roots, bark cells and wheat seedlings (germinated in the dark) during hardening to withstand freezing temperatures have shown either an increase in the degree of unsaturation of the fatty-acids, an increase in total weight of fatty-acid or an increase in weight of phospholipid (Gerloff, Richardson and Stahmann, 1966; Siminovitch, Rheaume, Pomeroy and Lepage, 1968; and De La Roche, Andrews, Pomeroy, Weinberger, and Kates, 1972). In relation to the hardening of plant leaves Kuiper (1970) found that the weights of MGDG, DGDG, PC, and PE increased in alfalfa leaves grown at 15°C in comparison to leaves at 30°C but that differences in the fatty-acid composition of the lipids were negligible. However, Kuiper does not state whether the plants grown at the lower temperature were more chill or frost-resistant. Since 15°C is a high temperature for hardening against frost-injury it is thought that the increased levels of the above lipids at this temperature may

Table 2. Increases in the unsaturation of fatty-acids during the acclimatization of organisms to chilling and freezing temperatures

Species	Fatty-acid or lipid fraction analysed	Acclimatization Temperature °C	Reference
Plants:			
Escherichia coli	Total fatty-acid	10.0	Marr & Ingraham (1962)
Anacystis nidulans	Total fatty-acid	26.0	Holton, Blecker & Onore (1964)
Cyanidium caldarium	Phospholipid and glycolipid	20.0	Kleinschmidt & McMahon (1970)
Chlorella sorokina	Total fatty-acid	22.0	Patterson (1970)
Medicago sativa	Total fatty-acid of the roots	-2.5	Gerloff, Richardson & Stahmann (1966)
Triticum aestivum	Total fatty-acid of seedlings	2.0	De La Roche, Andrews, Pomeroy, Weinberger & Kates (1972)
Populus euramericana	Phospholipid of stem bark	0.0	Yoshida & Sakai (1973)
Hordeum vulgare	Phospholipid of the leaves	12.0	Wilson & Crawford (1974) (present thesis)
Gossypium hirsutum	Phospholipid of the leaves	12.0	Wilson & Crawford (1974) (present thesis)
Phaseolus vulgaris	Phospholipid of the leaves	12.0	Wilson & Crawford (1974) (present thesis)

Table 2. (continued)

Species	Fatty-acid or lipid fraction analysed	Acclimatization Temperature °C	Reference
Animals:			
Crustacean plankton	Total fatty-acid	2.8	Farkas & Herodek (1964)
Calliphora erythrocephala (blow-fly)	Phospholipid	12.0	Fraenkel & Hopf (1940)
Lampito mauritti (earthworm)	Phospholipid	20.0	Nayeeunnisa (1966)
Carassius auratus (goldfish)	Total fatty-acid of the mitochondria	10.0	Caldwell & Vernberg (1970)
Rana esculenta (frog)	Phospho and neutral lipid of liver and adipose tissue	7.0	Barańska & Wlodawer (1969)
Mesocricetus auratus (hamster)	Total fatty acids of sub-cutaneous fat	6.0	Kodama & Pace (1963)
Rangifer tarandus (reindeer)	Total fatty acids of marrow fat	12.0	Irving, Schmidt-Nielsen & Abrahamsen (1957)

be correlated with increased resistance of these plants to chilling temperatures, although Rodniov, Nyuppieva and Zakharova (1973) detected slight increases in the weights of PC and PG during the hardening of potato leaves against freezing-injury by growing them for 3-4 days at 0-2°C. Unfortunately the latter authors did not analyse the fatty-acid composition of the lipids. In contrast, all that is known about the changes in the fatty-acid composition of chill-sensitive leaves in relation to hardening is that the weight of lipid soluble phosphorous decreased on hardening Gossypium hirsutum to withstand chilling at 5°C (Guinn, 1971).

A major problem in the elucidation of the factors which control the temperature at which the phase change takes place is that the precise nature and lipid composition of plant membranes is not known due to problems of isolation and purification. Only recently has a thorough study of the lipid and fatty-acid composition of pure plant mitochondria been possible (Schwertner and Biale, 1973). Table 3 compares the lipid composition of plant mitochondria, chloroplast and leaf preparations with that of bovine heart mitochondria. In general, plant membranes differ from animal membranes in having lower amounts of phospholipids and larger amounts of neutral and glycolipids. For example, phospholipids constitute over 94 per cent of the total lipids of bovine heart mitochondria in comparison to only 54 per cent in plant mitochondria and 10 per cent in chloroplasts. In plant mitochondria a significant neutral and glycolipid fraction is present and in chloroplasts

Table 3 . Classification of lipids and estimates of percentage lipid composition of some membrane components isolated from plant and animal tissues [a, b]

Lipid	Animal	Plant		
	Bovine heart mitochondria[c]	Cauliflower (Brassica oleracea) mitochondria[d]	Spinnach (Spinacea oleracea) Chloroplasts[e]	Kidney Bean (Phaseolus vulgaris) Total Leaf[f]
Polar Lipids				
Phospholipids:				
Phosphatidyl choline	38.7	17.8	3.9	9.1
Phosphatidyl ethanolamine	35.0	21.1	0.2	4.4
Phosphatidyl inositol	3.1	5.4	1.3	1.6
Phosphatidic acid	-	1.6	-	5.3
Phosphatidyl glycerol	-	2.2	4.6	1.2
Diphosphatidyl glycerol	17.9	3.2	trace	-
Phosphatidyl serine	trace	2.7	-	-
Total Phospholipids	94.7	54.0	10.0	21.6
Glycolipids:				
Monogalactosyl diglyceride	trace	2.2	30.0	22.4
Digalactosyl diglyceride	trace	2.7	15.0	14.2
Sulpholipid	-	1.3	5.0	1.3
Total Glycolipids	trace	6.2	50.0	37.9
Total Neutral Lipids*[g]	6.2	18.9	28.2	40.5

*Neutral Lipids include: Sterols, Sterol esters, Mono, di and tri-glycerides, and free fatty-acids.

a The calculations used in the preparation of this table have often required the making of assumptions that could not be verified from the papers from which the data were drawn. Because of this, and because of the variations in experimental results, the reader is advised that the tabulated values should be considered only as estimates presented to emphasize the differences between plant and animal membranes.

b The data have been calculated as the percentage by weight of the total weight of lipid.

c Dewey and Barr, 1970.

d Schwertner and Biale, 1973.

e Kates, 1970.

f Results of present author.

g The weights of sterols and sterol esters are not always included in the estimate of total neutral lipid. However, the amount of these compounds in plant tissue is low (Davis and Finkner, 1972), especially cholesterol, so that the values given here are a realistic estimate of the neutral lipid fraction.

A dash (-) indicates that the analysis was not determined.

glycolipids constitute approximately 50 per cent of the total lipids. Since it is not possible to obtain pure mitochondrial preparations from green leaf tissue due to contamination by chloroplast fragments most research on chilling-sensitivity in plants has been performed on either roots, storage organs, hypocotyls or fruits. Therefore,the effects of chilling on the lipids of leaf membranes have received less attention, although the most striking visual effects and perhaps the most important ecological effects (in terms of range of survival of species), occurs when the leaf is exposed to chilling temperatures. However, the problems of membrane isolation from leaf tissue are not as great as is sometimes suggested when it is remembered that the majority of cellular lipids and their associated fatty-acids are membrane bound, with the exception of the seed oils and the waxy cuticles of leaves (Kates, 1970). Leaf analyses (present author) have shown that over 60 per cent of the total leaf fatty-acid is associated with the polar lipids (Table 3), which are considered to be found almost exclusively within the membranes.

Investigations on the changes in lipid and fatty-acid composition during the acclimatization of plants and animals to low temperatures have shown increases in the degree of unsaturation of either the total fatty-acid of the tissue analysed, or an increase in unsaturation of the phospholipids (Table 2). However, the majority of these studies only follow the changes in the fatty-acid composition of a single species and omit the changes which might occur in cold-tolerant organisms at the same temperature or when the organism is chill-damaged. Because leaf membranes contain less phospholipid and more neutral and glycolipid than animal membranes it was not clear

whether the temperature at which the phase change occurred in chill-sensitive plants was determined by the overall degree of unsaturation of the lipid matrix or specific lipid components. Therefore, it was decided to investigate the changes in the degree of unsaturation and weight of the total leaf fatty-acids and polar lipids on hardening and chilling several chill-sensitive species. These changes were compared to those which occurred in chill-resistant species at the same temperatures and those chill-sensitive species in which hardening was not effective. As a further control a comparison was also made of the changes in weight and fatty-acid composition of the polar lipids of chill-sensitive and resistant plants with increase in physiological ate at 25°C and this was examined in relation to the increased susceptibility of older leaves to chilling-injury.

Preliminary investigations studied the metabolic consequences of the phase change in the lipid layer of the mitochondrial membranes of chill-sensitive plants during chilling.

This study has tried to find answers to the following questions:-

1) Is chilling-injury in leaves caused mainly by dehydration or metabolic imbalances as a result of the phase change in the lipid layer of the cellular membranes?

2) Are the membrane fatty-acids of chill-resistant plants grown at 25°C more unsaturated than chill-sensitive plants grown at 25°C?

3) To what extent is it possible to harden chill-sensitive plants against chilling-injury at 5°C, 85 per cent RH?

4) Does the degree of unsaturation and weight of membrane lipids of chill-sensitive species increase on hardening over 4 days at 12°C? Do these changes occur in all membrane lipids? Do similar changes occur in chill-sensitive plants in which the hardening treatment is ineffective and in chill-resistant plants grown at the same temperatures?

5) How do the changes in the fatty-acid composition of the membranes of chill-sensitive species during hardening prevent chilling-injury?

6) How do changes in the degree of unsaturation and weight of lipids on chilling chill-sensitive species affect the reversibility of the phase change?

7) Is the greater susceptibility of older leaves to chilling-injury related to changes in the fatty-acid composition of the membranes of chill-sensitive plants with increase in age?

8) Do membrane lipids provide a general matrix, the phase change of which is largely determined by its overall fatty-acid composition?

9) Are the changes in the lipid and fatty-acid composition of chill-sensitive species during hardening to chilling temperatures similar to those on hardening chill-resistant species to sub-zero temperatures?

10) Does a phase change in the membrane lipids occur at sub-zero temperatures in chill-resistant species and thus hasten the speed at which freezing-injury occurs?

CHAPTER ONE

THE PLANT MATERIALS

1. SUSCEPTIBILITY TO CHILLING-INJURY

Chill-sensitivity is largely confined to tropical and sub-tropical plants, although some temperate plants grown at high temperatures can also be susceptible to temperatures in the 0 to 10°C range. Therefore, in this country, seedlings of temperate species grown in a hot-house are often transferred to a cold frame for hardening before being planted outside in the Spring as this reduces susceptibility to chill and frost-injury. In the present study chill-sensitive species are defined as those plants of tropical and sub-tropical distribution which, when transferred from 25°C, 95 per cent RH, to 5°C, 85per cent RH, for 48 hours develop necrotic lesions on the leaves during chilling or on the subsequent return of the plants to 25°C, 50 per cent RH. Conversely, chill-resistant species are defined as those plants of temperate or arctic-alpine distribution which, when transferred from 25°C, 95 per cent RH to 5°C, 85 per cent RH for 48 hours do not develop decrotic lesion on the leaves or on return to 25°C, 50 per cent RH.

Susceptibility to chilling-injury depends on the following factors:-

1) The degree of tolerance of the species, i. e. the extent to which the species can withstand the metabolic changes on chilling without producing any visible injury.
2) The temperature at which the species is chilled. Some species e. g. Episcia reptans are injured at 12°C, whilst other species, e. g. Gossypium hirsutum are not injured until the temperature drops below 10°C.

3) The temperature of growth prior to chilling. Some chill-sensitive species e. g. Phaseolus vulgaris can be hardened to withstand chilling by growing them at a temperature slightly above the chilling range for 4 days before chilling. In contrast, an extremely chill-sensitive species, Episcia reptans, cannot be hardened against chilling-injury at 8°C, 85 per cent RH, by 4 days' growth at 15°C, 95 per cent RH, before chilling, the lowest temperature at which this species will grow without injury.

4) The physiological age of the leaf. In general, older leaves are more susceptible to chilling. However, Sellschop and Salmon (1928) found that the younger parts of a number of species tested were more susceptible to chilling than the more mature parts. The different results may be explained by the fact that the plants used in their investigation were grown in a well ventilated greenhouse before chilling. The present author has found that ventilation draughts caused older plants to harden and it is thought that the results of Sellschop and Salmon may have been influenced by such factors, especially as no mention is made of growth temperature before chilling.

5) The humidity at which the plant is chilled. Sachs (1864) found that tobacco, squash and bean plants kept at 2 to 4°C in glass jars to maintain high humidity suffered no chill-injury. In contrast, Molisch (1896, 1897) demonstrated that a large variety of plants died even if chilled at 100 per cent RH. In agreement with these results the present author has found that leaves of Phaseolus vulgaris could be chilled at 100 per cent RH (by enclosure in polythene bags) without incurring injury. Leaves of the very

chill-sensitive species Episcia reptans were, however, severely injured after 24 hours chilling at 5°C, 100 per cent RH, although the rate of development of injury was slower than during chilling at 5°C, 85 per cent RH. Therefore in some species (e. g. Phaseolus vulgaris) chilling-injury appears to be mainly dependent on water loss from the leaves, and in the more chill-sensitive species (e. g. Episcia reptans) chilling-injury is due to a combination of water loss and metabolic imbalances. Comparisons of the effects of humidity on the extent of chilling-injury found by different authors are made difficult by the fact that other factors such as wind speed and turbulence, which also influence the rate of water loss, are not known.

6) The light intensity. Leaves of Phaseolus vulgaris suffer 5 times less injury when chilled at 5°C in the dark for 24 hours in comparison to chilling at 71.6 W. M.$^{-2}$ (P. 45).

7) The mineral nutrition of the plant. Sellschop and Salmon (1928) found that plants watered with KNO_3 were more resistant to chilling-injury. This was attributed to a protective effect of K^+ ions on the plants but may also be related to the physiological age of the leaves. Buckwheat also shows increased resistance to chilling when the potassium supply is increased (Korovin and Frolov, 1968). It is interesting to note that K^+ is the major ion which leaks out of the leaf on chilling.

8) The growth conditions on return to the warmth. Since the severity of chilling-injury depends on temperature, humidity and light intensity, it is expected that these factors would influence the extent of injury when plants are returned to the warmth. In

the following experiments plants were returned to 25°C, 50 per cent RH and a light intensity of 14.6 W. M. $^{-2}$ after chilling.

2. THE PLANT SPECIES

To formulate general metabolic mechanisms by which plants are able to withstand environmental stresses it is necessary to study a wide range of species. In this investigation 21 species were used ranging from extremely chill-sensitive tropical and sub-tropical species to chill and frost-resistant temperate and arctic-alpine plants. The chill-sensitive plants can be divided into two categories based on their susceptibility to chilling-injury.

Chill-sensitive

Category One : Extremely chill-sensitive species which are rapidly damaged on exposure to chilling temperatures between 12 and 15°C and which cannot be readily hardened against chilling-injury. Maintaining 100 per cent RH during chilling at 5°C does not prevent injury to these species, although the speed at which injury occurs is reduced. Therefore, it is suggested that metabolic imbalances are the main pathway leading to injury to these species and that water loss may be of secondary importance.

Episcia reptans. Mart. Gesneriaceae. Brazilian origin.

Nautilocalyx lynchii[ab]. Sprague. Gesneriaceae. Colombia.

---

a Species not tested for ability to harden.

b Species not tested for temperature at which injury first occurs.

Additional species which may be included in this category but which were not used in the present investigation include:-

Species injured at 12°C

Saccharum officinarum[ac]. (Faris, 1926).

Oryza sativa[ac]. (Adir, 1968; Tsunoda, Fujimura, Nakahari and Oyamado, 1968).

Species in which injury cannot be prevented by maintaining 100 per cent RH. From 28 species listed by Molisch (1897).

Episcia bicolor[ab]. Hook. Gesneriaceae.

Eranthemum tricolor[ab]. Nichols. Acanthaceae.

Eranthemum couperi[ab]. Hook. Acanthaceae.

Boehmeria argentea[ab]. Linden. Urticaceae.

Iresine acuminata[ab]. Amaranthaceae.

Uhdea bipinnatifida[ab]. Kunth. Compositae.

Eranthemum nervosum[ab]. R. Br. Acanthaceae.

Category Two : Chill-sensitive species which are damaged in the 0 to 10°C range. The majority of these species can be hardened against chilling-injury at 5°C, 85 per cent RH, by 4 days growth at 12°C, 95 per cent RH, before chilling. Maintaining 100 per cent RH during chilling at 5°C prevents injury to these species. Therefore it is suggested that water loss is the main pathway leading to injury in these species and that metabolic imbalances are of secondary importance.

a Species not tested for ability to harden.

b Species not tested for temperature at which injury first occurs.

c Species not tested for susceptibility to chilling-injury at 100 per cent RH.

Gossypium hirsutum[c]. L. cv. Westburn or Parrot. Malvaceae. Tropical American origin.

Phaseolus vulgaris. L. cv. Canadian Wonder. Leguminosae. American origin.

Cucumis sativus. L. cv. Telegraph. Cucurbitaceae. South Asian origin.

Saintpaulia grandiflora[c]. B. L. Burrt. Gesneriaceae. Tanganyika.

Synadenium grantii[a]. Hook. Euphorbiaceae. Tanganyika.

Coleus blumei[ac]. Benth. Labiatae. Java.

Ipomoea batatas[ac]. Lam. Convolvulaceae. Cultivated throughout the world tropics.

Zea mays.[ac] Graminae. Origin unknown.

Chill-resistant

Hordeum vulgare. L. cv. Golden Promise. Cultivated throughout the temperate regions of the world.

Beta vulgaris. L. Chenopodiaceae. Cultivated in Europe.

Brassica rapa. L. Cruciferae. Cosmopolitan cultivar.

Brassica oleracea. L. Cruciferae. Coasts of Western Europe.

Brassica capitata. L. Cruciferae. Coasts of Western Europe.

Saxifraga umbrosa. L. Saxifragaceae. Eastern Europe.

Saxifraga aizoides. L. Saxifragaceae. Boreal Arctic regions.

Ligustrum ovalifolium. Hassk. Oleaceae. Japan.

Peperomia magnoliaefolia. A. Dietr. Piperaceae. American origin.

Primula bulleyana. Forrest. Primulaceae. Yunnan province.

Cheiranthus cheiri. L. Cruciferae. Europe.

---

a, c - Please refer to previous page.

3. GROWTH CONDITIONS

Since the temperature of growth before chilling can influence the susceptibility of the plants to chilling, all chill-sensitive plants were grown under greenhouse conditions at approximately 25°C under mist spray. Chill-resistant plants were generally grown in a slightly cooler greenhouse (20-25°C) at ambient humidity. All the plants were grown in John Innes potting compost No. 2 and transferred to the growth cabinet at 25°C and approximately 95 per cent RH for several days before the start of the temperature treatments to allow for the adjustment of the plants to growth cabinet conditions. 'Weyco' growth cabinets supplied by Fisons Scientific Apparatus Ltd. were used in these investigations. Unless otherwise stated all plants were grown with a 6-hour dark period from 2300 hours to 0500 hours on the following day. Chilling and hardening treatments were started at 1100 hours.

Chill-sensitive plants were hardened against chilling-injury at 5°C, 85 per cent RH, by growing them for 4 days at 12°C and approximately 95 per cent RH at a light intensity of 71.6 W.M.$^{-2}$ before chilling. Because the plants wilted rapidly on transfer to 12°C, high humidity was maintained to reduce water loss and the susceptibility of the plants to chilling-injury due to the desiccation of the leaves. Growth cabinets maintained at 12°C were defrosted every 24 hours.

Because the degree of chilling-injury depends on temperature, humidity and light intensity, all plants were chilled at 5°C, 85 per

cent RH under an 18-hour photoperiod of 71.6 W.M.$^{-2}$ To maintain 85 per cent RH in the growth cabinets at 5°C it was necessary to defrost them quickly every 12 hours. After chilling the plants were returned to 25°C and approximately 50 per cent RH at a light intensity of 14.6 W.M.$^{-2}$ Unfortunately the plants could not be returned to a light intensity of 71.6 W.M.$^{-2}$ due to the different lighting conditions in the growth cabinets available for this investigation.

Further details of the species used, their physiological age and growth conditions are given at the beginning of each chapter. Table 4 summarizes the temperature and humidity treatments used in this investigation.

4. SYMPTOMS OF CHILLING-INJURY

The symptoms of chilling-injury in leaves are often visible after only a few hours chilling, whereas in fruits damage does not usually become apparent until several days or weeks at the chilling temperature. Therefore chilling-injury is a function of physiological injury per se (i.e. a combination of metabolic disorders and desiccation) and the rate of symptom development.

The development of chilling injury in leaves of 15-day-old plants of Cucumis sativus is shown in plates 1-4 and follows a similar pattern in Phaseolus vulgaris and Gossypium hirsutum, the main chill-sensitive species used in this study. After only 4 hours chilling at 5°C, 85 per cent RH, the leaves are wilted (Plate 1), the wilting becoming more severe with increased

Table 4. Temperature and humidity treatments used in this investigation and their effects on chill-sensitive plants

Treatment	Temperature and humidity	Species	Effect
1) Controls	25°C, 95 per cent RH	All species (11 chill-sensitive species used, see P. 21)	-
2) Hardening	12°C, 95 per cent RH	Phaseolus vulgaris Gossypium hirsutum Cucumis sativus Saintpaulia grandiflora	Hardening effective against chilling at 5°C, 85 per cent RH
	12°C, 100 per cent RH	Phaseolus vulgaris	Hardening not effective against chilling at 5°C, 85 per cent RH.
	15°C, 95 per cent RH	Episcia reptans	Hardening not effective against chilling at 8°C, 85 per cent RH.
3) Chilling	5°C, 85 per cent RH	All species	Severe injury
	5°C, 100 per cent RH	Phaseolus vulgaris	No injury after 4 days chilling at 100 per cent RH. Plants not hardened against subsequent chilling at 5°C. 85 per cent RH.
	5°C, 100 per cent RH	Episcia reptans	Severe injury after 24 hours chilling at 5°C, 100 per cent RH
	12°C, 85 per cent RH	Episcia reptans	Severe injury
4) Return to warmth after chilling	25°C, 50 per cent RH	All species	-

length of exposure to chilling. Light green patches usually develop after 7 hours chilling thus indicating chlorophyll loss. Necrotic areas may also be visible at this time (Plate 2) or, if not immediately conspicuous, develop after transfer to 25°C for 24 hours. The rapid decreases in the fresh weight of the leaves of chill-sensitive Phaseolus vulgaris and Gossypium hirsutum are shown in Fig. 1 in comparison to the small decrease in fresh weight on chilling chill-resistant Hordeum vulgare. Curling and drying of the leaf margin of the chill-sensitive species occurs after 24 hours chilling (Plate 3).

When the plants are returned to 25°C, 50 per cent relative humidity, the leaf regains turgor within 12 hours and after 24 hours large brown or white necrotic lesions develop (Plate 4). If the plants are chilled longer than 24 hours the severity of injury increases, the stems wilt and turn brown resulting in the death of the plant. Necrosis appears more rapidly in both leaves and fruits when they are returned to a higher temperature after chilling so that estimates of chilling-injury are usually made after a period of recovery in the warmth. The lesions which occur as a result of chilling may allow the invasion of decay micro-organisms into the tissue leading to the subsequent death of the plant.

A common symptom of chilling-injury in leaves and fruits is external discolouration. Plate 5 shows that when Episcia reptans was chilled at 12°C for 48 hours the dark green variegated leaves turned brown and died. (In this plate it

can also be seen that the older, outer leaves are more susceptible to chilling-injury than the younger, inner leaves). Chilling Nautilocalyx lynchii results in the development of light red patches on the dark green leaves after 24 hours chilling (Plate 6). These patches usually remain light red for 12 to 14 days after the end of chilling and then turn brown and dry (Plate 7). In Coleus blumei the top 2 cms of the shoots are particularly sensitive to chilling-injury, turning brown and dying after 48 hours exposure to 5°C. The death of these shoots and the discolouration becoming visible only after 2 days recovery at 25°C.

The leaves of the chill-resistant species showed no signs of damage when transferred from 25 to 5°C. However, the leaves of 15-day-old seedlings of Beta vulgaris and Brassica rapa rapidly wilted on chilling for 48 hours but quickly regained turgor on return to 25°C with no signs of necrosis.

5. HARDENING IN THE LIGHT

Although it is well known that many temperate plants can be hardened to withstand sub-zero temperatures (Levitt, 1972) few attempts have been made to harden tropical and sub-tropical species against chilling temperatures. Most studies on hardening of chill-sensitive plants have been performed on the fruits of these species due to the commercial advantage of being able to prolong their market life by storage at low temperatures. Attempts at hardening fruits have met with little success (P. 3) and this led to the opinion that the leaves of chill-sensitive plants could not be effectively hardened

THE DEVELOPMENT OF CHILLING-INJURY IN 15-DAY-OLD CUCUMIS SATIVUS PLANTS DURING CHILLING AT 5°C, 85 PER CENT RH, FOR 24 HOURS AND ON RETURN OF THE PLANTS TO 25°C, 50 PER CENT RH (Controls on the left, chilled plants on the right).

Plate 1. Chilled 4 hours. Plants start to show signs of wilting.

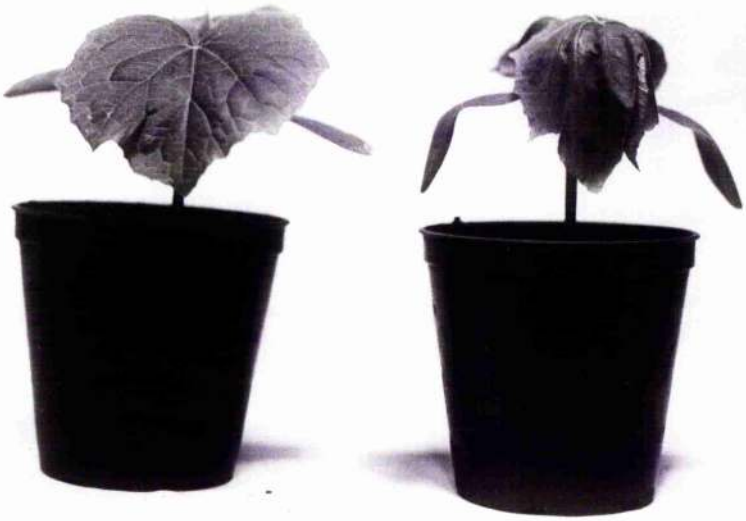


Plate 2. Chilled 7 hours. Wilting has increased and light green or necrotic patches may be visible.

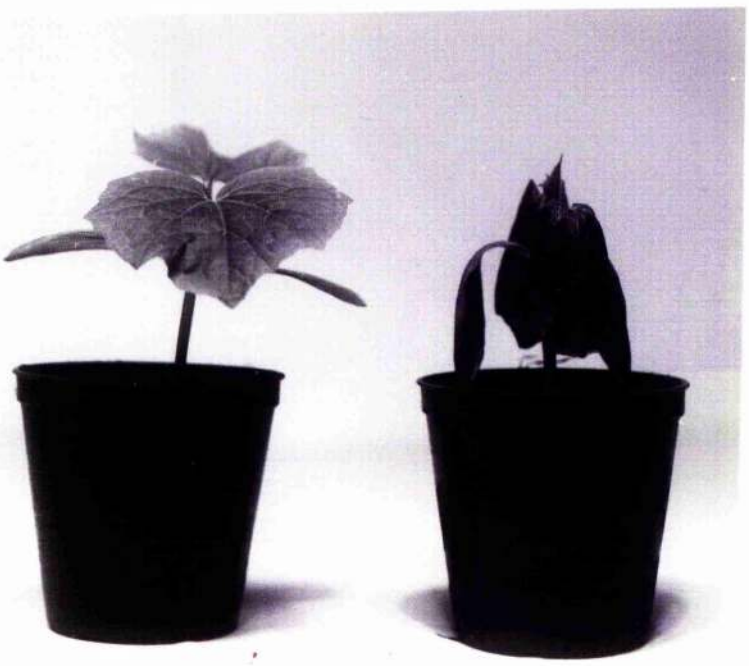


Plate 3. Chilled 24 hours. Wilting is now severe, the area of necrosis has increased and damage due to desiccation is suggested by the drying of the leaf edge.

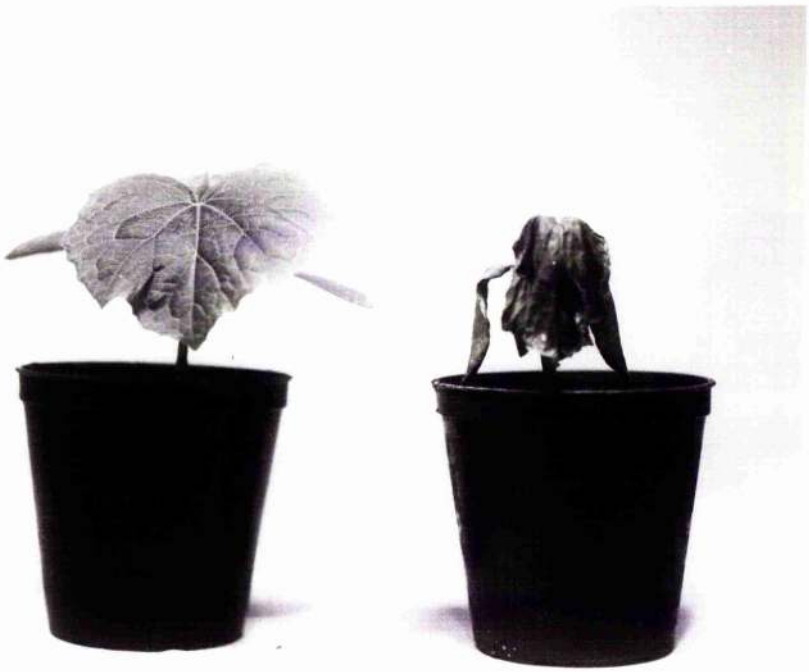
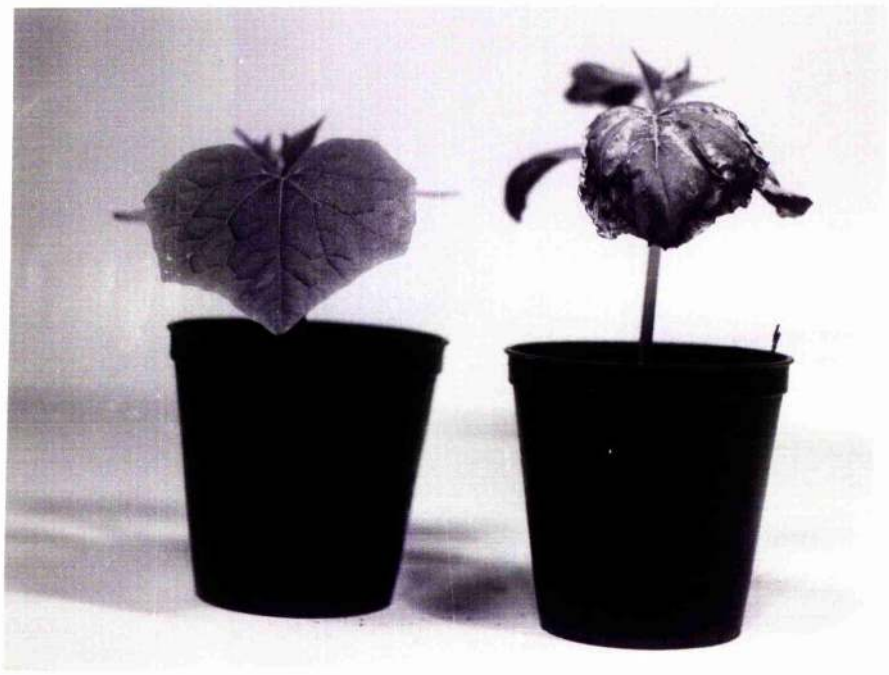


Plate 4. 24 hours recovery at 25°C, 50 per cent RH. The leaves quickly regain their turgor and large brown or white necrotic lesions develop. The leaf margin dries and becomes brittle.



THE DEVELOPMENT OF CHILLING-INJURY IN EPISCIA REPTANS

Plate 5. The plant on the right was chilled for 48 hours at 12°C, 85 per cent RH, and then returned to 25°C, 50 per cent RH, for 48 hours. In the chilled plant the dark green variegated leaves turned brown and died. It can also be seen that the older, outer leaves are more susceptible to chilling-injury than the younger, inner leaves.

Plate 6. The development of light red patches on the leaves of Nautilocalyx lynchii on return to 25°C after 24 hours chilling at 5°C, 85 per cent RH. (Chilled plant on the left, control plant on the right).

Plate 7. The light red patches on the leaves of Nautilocalyx lynchii due to 24 hours chilling at 5°C, 85 per cent RH, eventually turn brown and dry after 35 days recovery at 25°C. (Plant on the left has had 12 days recovery at 25°C, the plant on the right 35 days recovery).

(Lyons, 1973). However, in this study it has been found that a period of 4 days at 12°C, 95 per cent RH, and 18-hour photoperiod of 71.6 W.M.$^{-2}$ provided protection against 48 hours chilling at 5°C, 85 per cent RH, in the leaves of Gossypium hirsutum, Phaseolus vulgaris, Cucumis sativus and Saintpaulia grandiflora. Two days is the minimum time in which hardening can be achieved, the plants incurring injury if chilled after only 1 day at 12°C. In contrast, the leaves of some species do not harden with the same facility as the above species. Episcia reptans will only harden after several months at hardening temperature (see P. 3). The effectiveness of the hardening treatments used in this investigation are discussed in detail on P. 38.

The transfer of the chill-sensitive plants Gossypium hirsutum, Phaseolus vulgaris and Cucumis sativus from 25 to 12°C, 95 per cent RH resulted in severe wilting of the leaves after 12 hours. However, leaf turgor was regained after 24 to 48 hours at 12°C, 95 per cent RH.

When the hardened plants were subsequently chilled the leaves did not wilt as quickly as the non-hardened plants. Only after 7 hours chilling did wilting become noticeable in the hardened plants. Fig. 1 shows that hardening plants of Gossypium hirsutum and Phaseolus vulgaris reduced water loss by approximately 10 per cent during the first 12 hours chilling. By the end of 24 hours' chilling the water loss of the hardened plants was approximately 30 per cent less than that of the non-hardened leaves. This was reflected in the severity of wilting. The leaves of the non-hardened

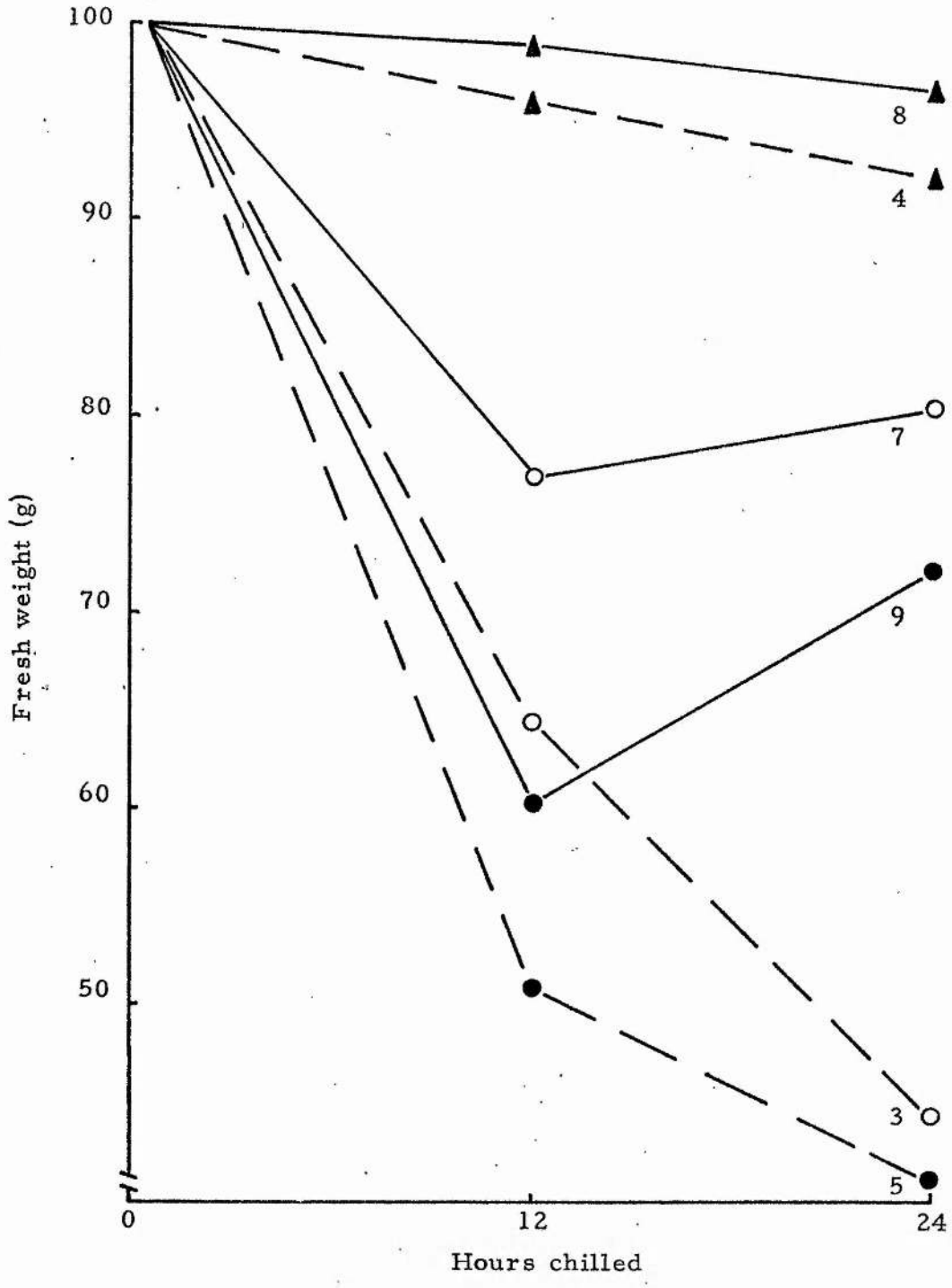


Fig. 1. Changes in the fresh weight of hardened and non-hardened leaves on chilling at 5°C, 85 per cent RH, in the light. Continuous lines indicate plants which had been hardened for 4 days at 12°C, 95 per cent RH in the light before chilling, and broken lines plants which remained at 25°C before chilling. The figures beside the points refer to the age of the leaves at the start of the chilling treatments. Leaves were grown under growth-cabinet conditions of 25°C and 95 per cent RH before chilling.

● Gossypium hirsutum; ○ Phaseolus vulgaris; ▲ Hordeum vulgare.

plants became increasingly wilted during 24 hours' chilling in contrast to the leaves of the hardened plants which had regained a large amount of their turgor by the end of the chilling treatment. The increase in turgor of the hardened leaves is shown by the increase in fresh weight of the leaves after 24 hours' chilling in comparison to the fresh weight after 12 hours' chilling (Fig. 1).

The wilting of the non-hardened chill-sensitive plants on chilling could be due to 3 factors:-

1) An increase in membrane permeability. The phase transition in the membrane lipids at chilling temperatures is considered to result in an increase in membrane permeability (P. 5).
2) A reduction in the rate of translocation. Giaquinta and Geiger (1973) concluded that chilling may inhibit translocation in chill-sensitive species by causing the lipid portion of the plasma membrane or perhaps the sieve tube reticulum to undergo a phase change.
3) The opening of the stomata on chilling. Wright (1971) found that chilling leaves of Cucumis sativus resulted in the initial closure of the stomata followed by opening after 3 hours' chilling.

Because hardening is thought to lower the transition temperature of the membrane lipids so that the phase change does not occur on chilling (P. 191) the initial wilting of the hardened plants on chilling may be mainly due to the opening of the stomata. After 3 to 12 hours chilling the stomata may close in the hardened plants so that their leaves regain most of their turgor after 24 hours chilling (Fig. 1).

In addition, hardening was not 100 per cent effective against chilling-injury so that some water loss can also be attributed to a phase change in small areas of the membranes which were not completely hardened.

Hardening reduced the rate of water loss from the leaves of Gossypium hirsutum and Phaseolus vulgaris on chilling and may therefore have prevented injury due to desiccation of the leaf. In the leaves of chill-resistant Hordeum vulgare there was only a small decrease in the fresh weight of the leaves of the hardened and non-hardened plants on chilling (Fig. 1).

6. HARDENING IN THE DARK

In the previous section it was shown that several chill-sensitive species could be hardened against chilling-injury when grown under an 18-hour photoperiod of 71.6 W.M.$^{-2}$ at 12°C, 95 per cent RH. Low temperature in the absence of light has been shown to be less effective in inducing hardening against chilling-injury in leaves of Pisum sativum (Kuraishi, Arai, Ushijima and Tazaki, 1968). In addition, low temperature and light is generally required for the hardening of winter annuals, biennials and seedlings of perennials against freezing-injury (Levitt, 1972, P. 93). Therefore, it was decided to investigate whether 3-day-old leaves of Phaseolus vulgaris could be hardened in the dark at 12°C, 95 per cent RH, against chilling-injury at 5°C, 85 per cent RH. Because detrimental changes in chlorophyll and protein content of the leaves may occur due to a 4-day period in the dark (Wright and Simon, 1973), the plants were hardened for only 2 days at 12°C - a time period which achieved 95 per cent hardening in the light (P. 40). The effectiveness of hardening in the dark was tested by chilling the plants in the light

and the dark for 24 hours at 5°C, 85 per cent RH.

A) Plants hardened in the dark and subsequently chilled in the light

Three-day-old leaves of Phaseolus vulgaris hardened for 2 days in the dark at 12°C and subsequently chilled in the light for 24 hours suffered 74 per cent injury to the total leaf area - as shown by the degree of necrosis (Table 5). In contrast, leaves hardened in the light for 2 days and then chilled for 24 hours only suffered 5 per cent injury to the total leaf area (Table 5). Therefore, hardening at 12°C, 95 per cent RH, in the dark was not effective against chilling-injury at 5°C, 85 per cent RH, in the light.

B) Plants hardened in the dark and subsequently chilled in the dark

Leaves of Phaseolus vulgaris hardened for 2 days in the dark and then chilled for 24 hours in the dark only suffered 18 per cent injury to the total leaf area (Table 5). Similarly, chilling 3-day-old leaves of Phaseolus vulgaris for 24 hours in the dark, without any previous hardening treatment, reduced the extent of injury to the leaves to 17 per cent (see P. 45).

Therefore hardening against chilling and freezing-injury generally requires low temperature and light. Possible explanations for the inability of chill-sensitive plants to harden in the dark are discussed on page 181.

Table 5. The effectiveness of hardening 3-day-old leaves of Phaseolus vulgaris at 12°C in the light and the dark against subsequent chilling for 24 hours at 5°C, 85 per cent RH, in the light and the dark. Leaves were grown under growth-cabinet conditions of 25°C, 95 per cent RH for 3 days before the start of the temperature treatments. Damage estimated as the percentage of the total leaf area turning necrotic. (Values shown are the mean of 25 or more leaves).

Hardening treatment	Percentage of total leaf area damaged on subsequent chilling for 24 hours at 5°C, 85 per cent RH	
	Chilled in the light	Chilled in the dark
None	82.0	17.0
Two days at 12°C, 95 per cent RH in the dark.	74.0	18.0
Two days at 12°C, 95 per cent RH in the light.	5.0	Not determined
Four days at 12°C, 100 per cent RH in the light.	82.0	Not determined

7. THE EFFECTS OF 100 PER CENT RELATIVE HUMIDITY ON CHILLING-INJURY AND HARDENING

Leaves of Phaseolus vulgaris could be protected against chilling-injury over 4 days at 5°C by enclosing the plants in polythene bags, thus maintaining 100 per cent RH. Similarly, Wright and Simon (1973) found that the leaves of Cucumis sativus could be protected against chilling-injury by maintaining 100 per cent RH. The leaves of Phaseolus vulgaris remained turgid during chilling at 100 per cent RH but wilted rapidly on transfer to 25°C, 50 per cent RH, at the end of 4 days' chilling. Nevertheless, the leaves regained most of their turgor after 2 hours at 25°C and only a few small patches of necrosis could be detected on the leaves. This result support the theory that the phase change in the membrane lipids is completely reversible on return of the plants to the warmth if no degenerative injury has occurred (P. 5).

In contrast to the leaves of Phaseolus vulgaris, the leaves of the extremely chill-sensitive species, Episcia reptans, could not be protected against chilling-injury at 5°C by maintaining 100 per cent RH, although the rate of development of injury was slower than at 5°C, 85 per cent RH. Table 6 compares the rate of development of injury in Episcia reptans at 85 and 100 per cent RH on the turgor of the leaves and the extent of leaf discolouration. Although the brown, necrotic patches developed more slowly during chilling at 100 per cent RH, these patches developed rapidly on return to 25°C.

The leaves of Phaseolus vulgaris can be hardened against chilling-injury at 5°C, 85 per cent RH, by 4 days' growth at

Table 6. The rate of development of chilling-injury at 5°C in leaves of Episcia reptans during chilling at 85 and 100 per cent RH. Injury is based on leaf turgor and discolouration from green to brown

Hours chilled	5°C, 85 per cent RH	5°C, 100 per cent RH
24	Majority of leaves very wilted and turned brown.	A few leaves flaccid but no brown patches.
36	Increasingly injured, leaves curled.	Most leaves now flaccid. No brown patches.
48	Severely injured.	Brown patches now present on the leaves and injury is obvious.

12°C, 95 per cent RH, in the light before chilling (P. 29). Because the leaves of this species can also withstand chilling at 5°C, 100 per cent RH for 4 days, it might be expected that these plants would harden over this period and be able to withstand subsequent chilling at 5°C, 85 per cent RH. However, leaves of Phaseolus vulgaris held at 5°C for 4 days at 100 per cent RH, were found to be as susceptible to chilling-injury as leaves transferred directly from 25 to 5°C, 85 per cent RH (Table 5). In addition, hardening at 12°C, 100 per cent RH, for 4 days was not effective against subsequent chilling at 5°C, 85 per cent RH. The leaves of plants hardened at 12°C, 100 per cent RH for 4 days wilted rapidly on chilling at 5°C, 85 per cent RH, in a similar manner to plants hardened at 12°C, 95 per cent RH (Fig. 1). However, in contrast to the plants hardened at 12°C, 95 per cent RH, the leaves of the plants hardened at 12°C, 100 per cent RH, had not regained any turgor by the end of 24 hours' chilling. Table 5 shows that leaves of Phaseolus vulgaris held at 12°C, 100 per cent RH, for 4 days suffered the same amount of damage on chilling at 85 per cent RH as leaves transferred directly from 25 to 5°C, 85 per cent RH. The reasons for the inability of chill-sensitive plants to harden at 5 and 12°C, 100 per cent RH, by enclosure in polythene bags are discussed on page 180.

8. ESTIMATION OF CHILLING-INJURY

The most reliable method of estimating chilling-injury is on the visible symptoms described above. In this investigation two methods of estimating chilling-injury based on the degree of necrosis were used to determine, firstly, the effectiveness of the hardening treatments,

secondly, the effect of leaf age on the susceptibility of leaves to chilling-injury, thirdly, the variation in the susceptibility of individual leaves of the same age to chilling-injury, and finally, the effect of light intensity.

A) The effectiveness of hardening at 12°C, 95 per cent RH

In experiments on the total leaf fatty-acid composition (Chapter 3) the plants of Gossypium hirsutum, Phaseolus vulgaris, and Cucumis sativus were 56, 23 and 30 days old respectively and mature plants of Saintpaulia grandiflora were used. All these plants were grown under greenhouse conditions of approximately 25°C and 95 per cent relative humidity and, since they were available in large numbers, damage was estimated on the number of leaves showing injury and expressed as a percentage of the total number of leaves. Hardening these plants resulted in 7 to 22 per cent damage to the leaves (Table 7). The degree of damage to the hardened plants increased when they were subsequently chilled for 48 hours. However, most of the damage on hardening and chilling was confined to the lower, older leaves. Even though damage occurred to the hardened plants on chilling, table 7 shows that plants which had not been hardened before chilling suffered four times the amount of damage as the hardened plants. Damage was estimated after 1 to 10 days recovery at 25°C.

In subsequent experiments on the fatty-acid composition of the leaf polar lipids (Chapter 4) the leaves used were

Table 7. Percentage damage to plants (as shown by necrosis and curling of the leaves) on hardening at 12°C, 95 per cent RH and chilling at 5°C, 85 per cent RH in the light. Leaves were grown under greenhouse conditions of approximately 25°C and 95 per cent relative humidity before transfer to the growth cabinet. Damage was estimated as the number of leaves showing injury and expressed as a percentage of the total number of leaves.

Species	Age of plant (days)	Percentage damage due to hardening	Percentage damage to plants hardened and then chilled 48 hours	Percentage damage to non-hardened plants chilled 48 hours
Gossypium hirsutum	56	8.7	16.0	79.3
Phaseolus vulgaris	23	22.0	20.0	80.0
Saintpaulia grandiflora	nine months	7.0	33.0	100.0
Cucumis sativus	30	15.0	30.0	100.0

grown entirely under growth cabinet conditions at 25°C and 95 per cent relative humidity. Plants of Gossypium hirsutum were 18 days old and Phaseolus vulgaris 8 days old, and the leaves used were 5 days and 3 days old respectively. Hardening was more effective in the young leaves of these species grown under growth-cabinet conditions. Hardening 5-day-old leaves of Gossypium hirsutum and 3-day-old leaves of Phaseolus vulgaris for 4 days at 12°C produced very little damage to the young leaves of these plants and afforded 95 per cent protection against chilling-injury. Hardening 5-day-old leaves of Cucumis sativus for 4 days at 12°C also provided 95 per cent protection against chilling-injury. The younger leaves of these 3 species hardened more readily than the older leaves so that hardening was almost 95 per cent effective after only 2 days at 12°C.

B) The effect of leaf age on the susceptibility to chilling-injury

Since the above experiments had indicated that older leaves are more susceptible to injury than younger leaves, it was decided to compare the degree of chilling-injury to 5 and 9-day-old leaves of Gossypium hirsutum and 3 and 7-day-old leaves of Phaseolus vulgaris on chilling for 24 hours at 5°C. The leaves were grown under growth cabinet conditions and because these young plants had only one or two leaves the degree of chilling-injury was estimated as the percentage of the total leaf area which became necrotic out of a total of 25 or more leaves. Table 8 shows that the older leaves of both species were twice as severely damaged as the younger leaves after 12 hours chilling at 5°C. Damage was estimated after 48 hours recovery

Table 8. Percentage of total leaf area damaged (as shown by necrosis) due to chilling leaves of different ages at 5°C, 85 per cent RH, in the light. Leaves were grown under growth-cabinet conditions of 25°C and 95 per cent RH before chilling. (Values shown are the $\bar{X}$ of 25 or more leaves).

Species	Age of leaf (Days)	Percentage damage	
		Chilled 12 hours	Chilled 24 hours
Phaseolus vulgaris	3	54.0	82.0
	7	78.0	93.0
Gossypium hirsutum	5	48.0	65.0
	9	97.0	98.5

at 25°C. The greater degree of damage to the older leaves may be associated with the more rapid water loss of these leaves on chilling - as shown for the leaves of Phaseolus vulgaris in Fig. 2. Although the older leaves of plants are more susceptible to chilling-injury, young plants are often more susceptible to complete death by chilling due to the fleshy nature of their stems.

C) Variation in the susceptibility of individual leaves to chilling-injury

Leaves of the same species and age varied considerably in their susceptibility to chilling-injury. Table 9 shows that the degree of chilling-damage to individual leaves of Phaseolus vulgaris plants, due to 24 hours chilling at 5°C, varied between 0 to 100 per cent. Because leaves of the same age on a single plant also varied as widely in their susceptibility to chilling-injury, it is not thought that this variability was solely due to genetic differences between plants. Other factors such as the orientation of the leaf in relation to the direction of light and wind may also influence the susceptibility of the leaf to chilling-damage. Taylor and Rowley (1971) found that sorghum leaves placed horizontally to the direction of light were more chill-injured than the vertical leaves. The angle of the leaf will also be a significant factor influencing the rate of water loss from the leaf and injury due to desiccation. To minimise these

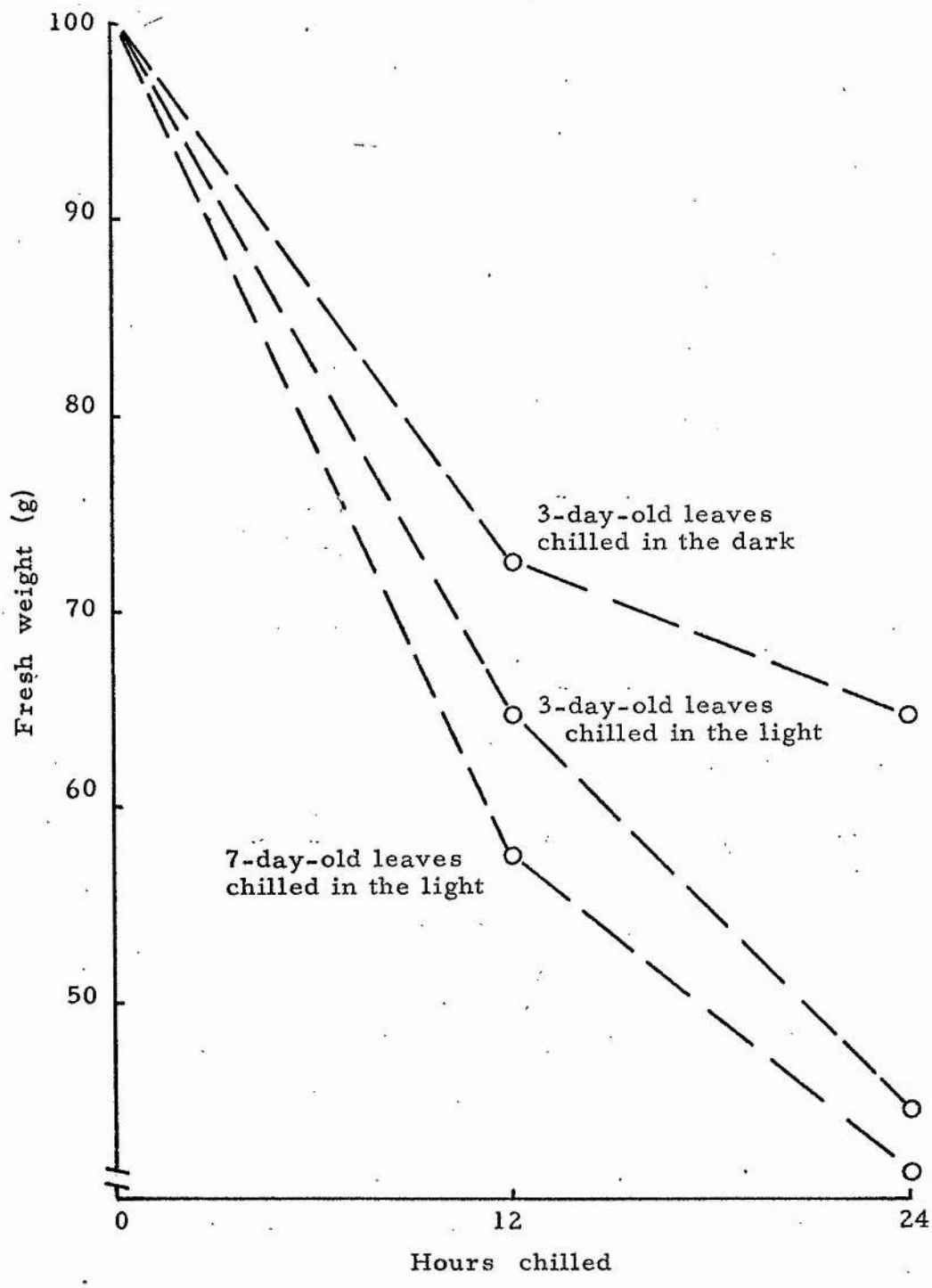


Fig. 2. The effect of light intensity and leaf age on the fresh weight of Phaseolus vulgaris leaves on chilling at 5°C, 85 per cent RH. Leaves were grown under growth-cabinet conditions of 25°C and 95 per cent RH before chilling.

Table 9. The variability in the susceptibility of individual leaves to chilling-injury. Percentage of the leaf area of individual 3-day-old leaves of Phaseolus vulgaris damaged by 24 hours chilling at 5°C, 85 per cent RH, in the light (as shown by the degree of necrosis). Leaves were grown under growth-cabinet conditions of 25°C and 95 per cent RH before chilling.

Plant number	Percentage damage	
	Leaf one*	Leaf two*
1	100	100
2	50	80
3	95	25
4	100	90
5	100	100
6	75	80
7	95	90
8	25	90
9	0	100
10	100	95
11	100	90
12	100	100
13	100	65
14	90	80
15	60	70

*The first two leaves on each plant of Phaseolus vulgaris are the same age.

effects the leaves were orientated in the same direction and mutual shading avoided as far as possible.

D) The effect of light intensity on the severity of chilling-injury

Leaves of sorghum (Taylor and Rowley, 1971) and cucumber (Kislyuk, 1964; Wright and Simon, 1973) have been shown to be less severely injured when chilled at low light intensities or in the dark. The effect of light intensity on chilling-injury was investigated further by chilling 3-day-old leaves of Phaseolus vulgaris at $5^{o}C$ for 24 hours in the dark and comparing them with leaves chilled in the light (71.6 $W.M^{-2}$).

The leaves of Phaseolus vulgaris wilted less rapidly when chilled in the dark and this is reflected in a reduction in fresh weight loss (Fig. 2). Also very few light green patches were visible at the end of 24 hours chilling which is consistent with the results of Wright and Simon (1973) who showed that chlorophyll was lost only from cucumber leaves if chilled in the light. After recovery at $25^{o}C$ only 17 per cent of the total leaf area was necrotic - a reduction in the extent of injury of approximately 5 times.

Therefore it appears that light enhances chilling-damage, perhaps due to time-dependent destruction of the photo-

synthetic apparatus. It is thought that when photosynthesis is inhibited that illumination can lead to cell damage through photochemical energy from chlorophyll being spent on photo-oxidation (Lomagin and Antropova, 1966). Injury may also be reduced on chilling in the dark by the closure of the stomata due to cessation of photosynthesis thus resulting in a decrease in fresh weight loss and a reduction in the extent of injury due to dehydration. However, Wright (1971) has shown that the stomata of cucumber leaves open after 3 hours' chilling in the light and the dark.

9. THE EFFECTIVENESS OF THE HARDENING TREATMENT AGAINST PROLONGED CHILLING AT 5°C, 85 PER CENT RH

Hardening chill-sensitive plants for 4 days at 12°C, 95 per cent RH, has been shown to provide protection against 48 hours chilling at 5°C, 85 per cent RH. If the chilling period is extended for 96 hours the hardened plants of Phaseolus vulgaris suffer very little extra damage. It is not known if the hardened plants have indefinite resistance to chilling at this temperature but it is probable that the cessation of growth, reduction in photosynthesis and the utilization of the cellular reserves of carbohydrate will eventually cause injury to the plant.

CHAPTER TWO

THE METABOLIC CONSEQUENCES OF CHILLING-DAMAGE TO THE MITOCHONDRIAL MEMBRANES OF CHILL-SENSITIVE PLANTS

INTRODUCTION

It has been shown that an immediate effect of chilling chill-sensitive plants and homeothermic animals is a phase change in the lipid layer of the mitochondrial membranes from a liquid-crystalline to a solid gel state (Lyons, 1972). These phase changes lead to the disruption of enzymic activity and are presumed to lead to the observed injury. Similar phase changes do not occur on chilling chill-resistant plants and poikilothermic organisms. However, if no degenerative injury occurs on chilling a chill-sensitive plant, such as when the chilling period is short, the phase change is immediately and completely reversible on return of the plants to the warmth, although the adjustment of the cellular metabolism back to the normal level for the higher temperature may take longer.

Permanent damage to the mitochondria on chilling can be expected to alter the respiratory pattern on return to the warmth. Research in relation to this aspect of chilling-injury has largely been confined to chill-sensitive fruits. Appleman and Smith (1936) found that the respiratory rate of chilled, chill-sensitive and resistant fruits on return to 22°C increased above the level of fruits held continuously at 22°C. Furthermore, an increase in respiration rate occurs in

chill-sensitive fruits on return to 25°C even when severely chill-injured (Eaks and Morris, 1956). The respiration rate increased to a plateau at the same time as the onset and development of chilling-injury and was followed by a decline at the time of death of the tissue.

The respiration rate of cotton leaves in relation to chilling-injury has been investigated by Amin (1969). He found that when cotton leaves were chilled for 12 hours at 2.8°C and then transferred to 25°C that the respiration rate increased above the normal level for 25°C, an effect similar to that which occurs in fruits. Creencia and Bramblage (1971) also showed that the respiration rate of leaf segments of Zea mays chilled for 36 hours at 0.3°C increased initially on return to 21°C and then declined to control level. The cotton and maize leaves used in these experiments suffered very little injury as a result of the chilling treatments. However, Amin (1969) showed that if cotton leaves were chilled for longer periods and became chill-injured, the respiration rate declined on return to 25°C. This effect is similar to the decline in respiration rate of chill-injured fruits at the time of death of the tissue. Therefore, it appears that an increase in respiration rate on return of the leaves to the warmth reflects that the phase change is almost completely reversible, resulting in only minor damage to the plant. Because research on the effects of chilling had centered on the respiration rate of chill-sensitive fruits and leaves on return to the warmth, it was decided to investigate the changes in respiration of the leaves of 5 chill-resistant plants and compare them with 4 chill-sensitive

species which differed in their susceptibility to chilling-injury.

The phase change in the lipid layer of the mitochondrial membranes of chill-sensitive plants increases the E_a of the membrane-associated enzymes. In contrast, soluble enzyme systems such as those involved in glycolysis proceed with a constant E_a over a range of 0 to 25°C (Raison, 1973). If the control processes which regulate the relative activities of the two systems are not sufficient to compensate for the change in rates, lowering the temperature below the point at which enzymes increase in E_a could result in major imbalances in metabolism. A result of these imbalances might be the accumulation of ethanol or acetaldehyde as the end products of glycolysis, or the accumulation of tri-carboxylic acids, perhaps to toxic levels, which may lead to the observed injury. Murata (1969) has detected increased levels of ethanol, acetaldehyde, pyruvate and α ketoglutarate in banana fruit pulp at 6°C over 15 days. In the same study he also found that malonate, a specific inhibitor of succinic dehydrodgenase, produced less inhibition of oxygen uptake in chilled tissue than in non-chilled tissue. Both these results indicate that tri-carboxylic acid cycle activity was affected to a greater degree by chilling temperatures than glycolytic activity. However, no information exists as to whether the same events occur in plant leaves. Therefore, it was decided to study the effect of malonate on the oxygen uptake of chilled and non-chilled leaves and to follow the changes in the levels of ethanol in chill-sensitive and resistant plants on chilling.

MATERIALS AND METHODS

1) Growth Conditions

The plants used in the experiments reported in this chapter were grown under greenhouse conditions as described on P. 24 and transferred to the growth cabinet for 2 to 4 days at 25°C before the start of the chilling treatment at 5°C, 85 per cent RH. All plants were several months old, except for plants of Cucumis sativus which were 30 days old at the start of the chilling treatment.

2) Respiratory Measurements

Species	Hours Chilled
Chill-sensitive	
Cucumis sativus	12
Nautilocalyx lynchii	24
Synadenium grantii	24
Coleus blumei	24
Chill-resistant	
Beta vulgaris	96
Cheiranthus cheiri	96
Primula bulleyana	96
Saxifraga umbrosa	96

Gas exchange measurements were made with a Gilson Respirometer over a 2-hour period on return to 25°C. The leaves were cut into discs of 55 sq mm with a cork borer, mixed, and 30 discs put into each flask containing 2 ml of phosphate buffer or buffer containing 0.1 M malonate. This concentration of malonate was used as Artemova and Zemlyanukhin

(1970) found it produced substantial inhibitions of oxygen uptake in maize and sunflower leaves and was not thought to produce non-specific effects due to the high levels of organic acids in leaves. The buffer was prepared as a 50/50, 0.05 M K_2HPO_4/ KH_2PO_4 solution. The pH of this solution was kept below 6 as it has been shown that malonate inhibition can disappear above pH 6. When malonate was added to the buffer solution the pH dropped to 2 and was adjusted to pH 4.5 with concentrated KOH. The buffer for the controls was adjusted to pH 4.5 with concentrated HCl. Oxygen uptake was measured in flasks with concentrated KOH in the central well. Duplicate samples of leaf gave highly reproducible results in all species and the respiration rate was constant after 20 minutes equilibration. Respiration measurements were made in the dark by enclosing the flasks in aluminium foil. Penetration of malonate into the leaf was achieved by vacuum infiltration. The flasks containing the leaf discs were placed in a vacuum desiccator and the whole evacuated twice, air being readmitted slowly each time. Experiments with controls showed that the vacuum treatment had no effect on the respiratory rate. Oxygen measurements were made over one hour and at the end of the experiment the leaf discs were dried at 100°C for 12 hours and the dry weight obtained.

3) Ethanol Concentration in the Leaves

The following species were used:-

Species	Hours Chilled
Chill-sensitive	
Nautilocalyx lynchii	18
Synadenium grantii	18
Chill-resistant	
Peperomia magnoliaefolia	18
Saxifraga aizoides	18

Samples of the chilled leaves were taken every two hours, placed in a muslin bag and crushed to extract the sap which was then centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was decanted and stored at -20°C until analysis. The sap was thawed slowly at 5°C and recentrifuged before analysis on a Pye Unicam gas-liquid chromatograph model 104 equipped with a hydrogen ion flame detector. Ethanol was separated from other compounds on a 1.52 m x 0.64 cm glass column packed with 20 per cent carbowax (polyethylene glycol) on diatomite 'C' (100-120 mesh). Nitrogen was used as the carrier gas at a flow rate of 35 ml per minute at a column temperature of 100°C. Ethanol was identified and quantified by the injection of a known standard. Because of the low amounts of ethanol in the sap, standards had to be injected both before and after each sap sample. The values shown in Fig. 3 are the mean of three sap samples.

RESULTS

1. THE EFFECT OF CHILLING ON THE RESPIRATION RATE OF CHILLED LEAVES ON RETURN TO 25°C

Chilling Cucumis sativus and Nautilocalyx lynchii for 12 and 24 hours respectively at 5°C produced twice as much damage to the leaves as the chilling of Synadenium grantii and Coleus blumei for 24 hours (Table 10). This difference in susceptibility to chilling-injury is reflected in the respiration rate of chill-sensitive plants on return to 25°C. In Cucumis sativus and Nautilocalyx lynchii, which were severely chill-injured, the respiration rate on return to 25°C was lower than that of plants held continuously at 25°C (Table 11). However, in Synadenium grantii and Coleus blumei, the species which were less chill-injured, the respiration rate on return to 25°C increased above the level for plants held continuously at 25°C during the first hour and showed signs of returning to the normal rate for 25°C over the second hour (Table 11).

An increase in respiration rate on return to 25°C also occurred in the chill-resistant leaves (Table 11), with the exception of Saxifraga umbrosa. The respiration rate of the chill-resistant leaves declined during the second hour at 25°C to a level nearer that of plants maintained continuously at 25°C.

If the chilling treatment had resulted in permanent damage to the mitochondria of chill-sensitive species, we might expect an increase in RQ on return of the plants to 25°C due to the relatively

Table 10 . Percentage of leaf area damaged (as shown by necrosis) due to chilling leaves of chill-sensitive plants

Species	Hours chilled at 5°C, 85 per cent RH	Percentage of leaf area damaged
Cucumis sativus	12	38
Nautilocalyx lynchii	24	53
Synadenium grantii	24	21
Coleus blumei	24	23

Table 11. The rate of oxygen uptake of chilled and non-chilled plant leaves at 25°C

Species	Non-chilled μl 0_2/100 mg dry wt/hour	Chilled μl 0_2/100 mg dry wt during 1st hour at 25°C	Chilled μl 0_2/100 mg dry wt during 2nd hour at 25°C	Percentage change in respiration of chilled leaves during the 1st hour at 25°C
Chill-sensitive :				
Cucumis sativus	286	236	225	-17
Nautilocalyx lynchii	147	101	92	-31
Synadenium grantii	134	167	159	+20
Coleus blumei	183	225	209	+23
Chill-resistant :				
Beta vulgaris	191	220	211	+13
Cheiranthus cheiri	176	209	191	+19
Primula bulleyana	306	400	391	+31
Saxifraga umbrosa	169	143	136	-15

All values shown are the $\overline{X}$ of duplicate samples.

greater increase in glycolytic activity than tri-carboxylic acid cycle metabolism. The RQ of Cucumis sativus increased from 1.6 to 2.0 on return to 25°C, which reflects that severe injury had occurred in this species resulting in a major upset in the normal metabolism of the tissue. In the other chill-sensitive and chill-resistant plants the RQ on return to 25°C was the same as that prior to chilling.

Northern populations of some plants have been shown to have higher respiration rates (Scholander and Kanwisher, 1959: Mooney and Billings, 1961), but no evidence was obtained for the thermal adaptation of the respiratory process in this study. Table 11 shows that there is considerable overlap in the respiration rates of temperate and tropical species maintained at 25°C.

2. THE EFFECT OF CHILLING ON THE RATES OF GLYCOLYTIC AND TRI-CARBOXYLIC ACID CYCLE ACTIVITY

A) The differential effect of malonate on the respiration of leaves before and after chilling

In both chill-sensitive and resistant plants the uptake of oxygen was decreased by the addition of malonate to the chilled and non-chilled leaves (Table 12). In the chill-resistant plants malonate produced the same percentage inhibition of oxygen uptake in the chilled and non-chilled leaves. However, in the severely chill-injured Cucumis sativus and Nautilocalyx lynchii malonate caused less inhibition of oxygen uptake than in the

Table 12. The effect of malonate on the inhibition of oxygen uptake by non-chilled and chilled plant leaves at 25°C

	Non-chilled			Chilled		
	μl 0_2/100 mg dry wt per hour		Percentage inhibition of oxygen uptake	μl 0_2/100 mg dry wt per hour		Percentage inhibition of oxygen uptake
	No malonate	With malonate		No malonate	With malonate	
Chill-sensitive :						
Cucumis sativus	286	47	83.0	236	102	57.0
Nautilocalyx lynchii	147	60	59.0	101	58	43.0
Synadenium grantii	134	114	15.0	167	148	12.0
Coleus blumei	183	69	62.0	225	104	54.0
Chill-resistant :						
Beta vulgaris	191	124	35.0	220	123	44.0
Cheiranthus cheiri	176	102	42.2	209	120	43.0
Primula bulleyana	306	139	55.0	400	160	60.0
Saxifraga umbrosa	169	81	52.0	143	70	51.1

All values shown are the $\bar{X}$ of duplicate samples.

non-chilled leaves, thus suggesting that the TCA cycle in these species was already adversely affected by the chilling treatment and that TCA metabolism did not increase to the normal rate for 25°C. In the chill-sensitive species Synadenium grantii and Coleus blumei there is only a small difference in the percentage inhibition of oxygen uptake of non-chilled and chilled leaves and this correlates well with the small amount of permanent damage to these species after 24 hours chilling.

B) Ethanol accumulation in chilled chill-sensitive plants

The level of ethanol in the leaves of chill-sensitive Nautilocalyx lynchii and Synadenium grantii increased to a maximum 6 hours after the start of the chilling treatment and then declined to the control level after 8 to 12 hours chilling (Fig. 3). This result is in contrast to that of Minchin and Simon (1973) who could not detect any increase in the level of ethanol in cucumber leaves after 3 days chilling at 5°C. Unfortunately, it is not clear whether they followed the changes in ethanol content over the 3-day period or only at the end of 3 days chilling. Therefore, the difference in results may be explained by the fact that Minchin and Simon only analysed cucumber leaves at the end of 3 days chilling and would not detect any transient increase during chilling.

The level of ethanol in the leaves of the chill-resistant species Peperomia magnoliaefolia and Saxifraga aizoides showed no change after 18 hours chilling (Fig. 3).

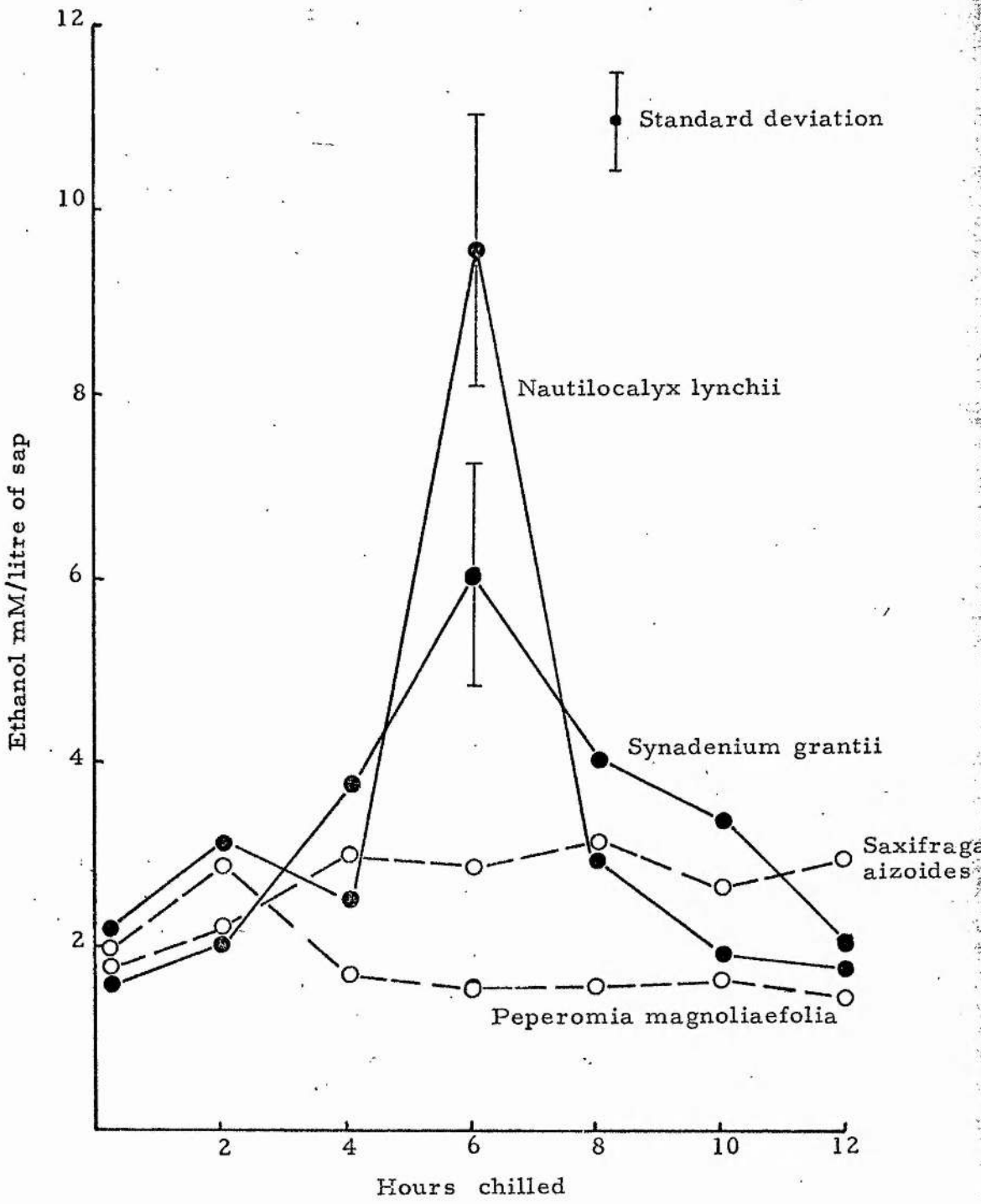


Fig. 3. Time course of changes in the ethanol content of leaves on chilling at 5°C, 85 per cent RH. Continuous lines indicate chill-sensitive plants and broken lines chill-resistant plants.

DISCUSSION

Chilling produced severe injury in the leaves of Cucumis sativus and Nautilocalyx lynchii resulting in death of the tissue and the depression of the respiratory rate on return to 25°C. However, one might expect that severe injury would result in a 'burst' of respiration due to the breakdown of membrane integrity, similar to that which occurs on wounding tissue. Presumably the 'burst' of respiration in leaves is small in comparison to the depression of mitochondrial respiration due to cell death. In chill-sensitive cucumber fruits which had been severely injured, Eaks and Morris (1956) found that the characteristic increase in respiration rate still occurred on return of the fruits to 25°C, although it later declined with the death of the tissue. This difference in behaviour between fruits and leaves could be due to the fact that symptoms of chill-injury develop more slowly in fruits or that there are greater cellular reserves of carbohydrate in fruits.

In those chill-sensitive species in which less injury occurred (Synadenium grantii and Coleus blumei), there was a transient increase in respiration rate on return to 25°C, perhaps due to the oxidation of metabolites such as ethanol which accumulate during chilling or the temporary uncoupling of oxidative phosphorylation. This increase in respiration rate on return to 25°C is similar to that reported for cotton leaves by Amin (1969). It could be speculated that in chill-sensitive leaves an increase in respiration

rate on return to 25°C may reflect the reversibility of the phase change whilst a decrease in respiration rate may reflect that permanent damage was caused by the chilling treatment. However, an increase in respiration rate on return to 25°C also occurred in the chill-resistant leaves, with the exception of Saxifraga umbrosa. These increases in the respiration rate of chill-resistant species on return to 25°C is thought to be due to an'over compensation' effect, the substrate for this transient increase being provided by the conversion of starch to sugars during chilling. The phenomenon of overshoot has been recorded in tomatoes by Went (1958), in chrysanthemum by Schwabe (1957) and in maize and sunflower leaves by Artemova and Zemlyanukhin (1970). In chill-resistant species low temperatures are known to favour the conversion of starch to sugar (Levitt, 1972) and this may also be the case in chill-sensitive species as Guinn (1971) showed that hardening increased the level of sugar in cotton leaves. Therefore, the causes of the increase in respiration rate of chill-sensitive and resistant plants on return to 25°C may be similar.

Malonate produced a lower percentage inhibition of oxygen uptake in the chilled leaves of Cucumis sativus and Nautilocalyx lynchii (Table 12). This result may have been due to the chilling treatment reducing the penetration of malonate into the leaf, perhaps by closing the stomata. However, a reduction in the penetration of malonate into the leaf due to chilling is thought to be unlikely as chilling results in a rapid increase in cell permeability

and opening of the stomata after 3 hours chilling at 5°C, 85 per cent RH (Wright, 1971). Even if the stomata closed in the chill-sensitive species on chilling, the penetration of malonate into the small leaf discs could easily be made via the cut edges of the disc, especially under vacuum infiltration. Furthermore, in the chill-resistant species the percentage inhibition of oxygen uptake due to malonate was the same in both the chilled and non-chilled leaves indicating that the chilling treatment had not affected the penetration of malonate.

The effectiveness of malonate is not the same in all species, as reflected by the different percentage inhibitions of oxygen uptake for each species (Table 12). Differences in the effectiveness of malonate may be due to variable rates of penetration of malonate between species and other factors, such as the amount of endogenous succinate and the ability of malonate to take an active part in metabolism.

The level of ethanol increased to a maximum 6 hours after the start of chilling in Nautilocalyx lynchii and Synadenium grantii and this coincides well with the time that chilling-injury (as shown by necrosis of the leaf) first becomes visible on return of the plants to 25°C. Thus the increase in the level of ethanol, although transitory, may lead to leaf necrosis. In contrast, the increase in the level of ethanol in banana fruit pulp is not transitory and increased over a 15-day period at 6°C (Murata, 1969). After 6 hours chilling the

level of ethanol in the leaves decreased, perhaps due to the control of glycolysis or the induction of pathways of conversion of ethanol into organic acids, amino acids, lipids or sugars. The existence of these pathways of interconversion in plant leaves has been shown by Cossins and Beevers (1963).

SUMMARY

The respiration rate of chilled chill-sensitive and resistant leaves increased on return to 25°C, except when the leaves were severely chill-injured. Further evidence for the greater inhibition of tri-carboxylic acid cycle activity than glycolytic activity on chilling chill-sensitive leaves was shown by the accumulation of ethanol after 6 hours chilling and the smaller inhibition of oxygen uptake by malonate in these leaves.

CHAPTER THREE

LEAF AND CHLOROPLAST FATTY-ACID CONTENT IN RELATION TO HARDENING AND CHILLING-INJURY

INTRODUCTION

Early investigators showed that the fats of animals (Pearson and Raper, 1927) and the seed oils of plants (Hilditch, 1956) originating from cold climates have a greater degree of unsaturation of the fatty-acids than plants or animals from the tropics. Further studies have suggested that the ability to withstand chilling-injury in both poikilothermic animals and chill-sensitive plants is associated with the high degree of unsaturation of the membrane fatty-acids, (Lyons, Wheaton and Pratt, 1964; Lyons, 1972). Recent research has cast doubts on whether the degree of unsaturation plays a dominant role in determining the temperature at which the phase change occurs in membranes. It has been demonstrated that when the unsaturated fatty-acid level of rat mitochondria was increased by dietary modification, this change had essentially no effect on the critical temperature (22-24^{o}C) of the phase change (Williams, Stancliff, Packer and Keith, 1972). Furthermore, studies by Long, Hruska and Gesser (1971) have shown that the presence of 50 mole per cent of cholesterol causes a gel to liquid-crystalline phase transition of bovine brain sphingomyelin at 20^{o}C. The addition of cholesterol to synthetic phospholipids was also found to lower the temperature of transition until at a third mole per cent the transition vanishes (Steim, Tourtellote, Reinert, McElhaney and Rader, 1969). Therefore it appears that cholesterol or other sterols could play an important role in determining the temperature at which phase

transitions occur in membranes and that the degree of unsaturation of the fatty-acids might not be as important as it was once considered (Lyons, 1972). However, Lyons (unpublished) has shown that dietary modification of the fatty-acid composition of sheep liver mitochondria did affect the transition temperature.

Fatty-acid analyses of mitochondria isolated from fruits have demonstrated that chill-resistant mitochondria have a greater degree of unsaturation of their fatty-acids than chill-sensitive mitochondria (Lyons et al., 1964). This correlation between the degree of unsaturation and chill-resistance was not found to be precise by Lyons and Asmundson (1965) and has been difficult to elucidate in the leaves of higher plants. Since it is not possible to obtain pure membrane or mitochondrial preparations from green leaf tissue due to contamination of the preparations by chloroplast fragments, the effects of chilling on the fatty-acids of leaf membranes and also the chloroplasts themselves have received less attention. The problems of membrane isolation from green leaf tissue are not so great as sometimes suggested when it is remembered that the majority of cellular lipids and their associated fatty-acids are largely membrane bound, with the exception of the seed oils and waxy cuticles of leaves (Kates, 1970). The present author has found that over 60 per cent of the total leaf fatty-acid is associated with the polar lipids (P. 14), which are considered to occur almost exclusively within the membranes. The neutral lipids, mainly mono- di- and tri-glycerides, comprise the remaining 40 per cent of the leaf fatty-acids (P. 14) and, according to the Danielli-Davson model of membrane structure, may be associated with membranes as a

layer with two monolayers of phospholipid on either side (Gurr and James, 1971, P. 193). Neutral lipids are, however, thought to be of secondary importance to the polar lipids as determinants of membrane structure. In addition to the above factors it was considered that total leaf fatty-acid analyses would give an accurate estimate of membrane fatty-acid composition, since it has been shown by spin labelling that a phase change occurs in crude preparations of chill-sensitive plants containing all the cell membranes, in fractions rich in mitochondria or chloroplasts and in micelles formed from lipids extracted from either whole tissue or from particular membranes (Raison, 1973). Because analyses of fatty-acids have so far been confined to mitochondria, it was decided to extend these investigations to the fatty-acid composition of the chloroplasts. A comparison was made of the leaf and chloroplast fatty-acid compositions of a large number of chill-sensitive and resistant plants grown at 25°C to determine whether chill-resistant plants have a higher degree of unsaturation of the membrane fatty-acids.

Changes in the lipid composition of plants on hardening to sub-zero temperatures have been well documented (P. 8). In contrast, practically no attempt has been made to identify the changes in fatty-acids and lipids which occur on hardening chill-sensitive species, with the exception of Guinn (1971) who reported a decrease in lipid soluble phosphorous on hardening Gossypium hirsutum. In the present study the changes in the degree of unsaturation and total weight of fatty-acid in the leaves and chloroplasts on hardening 4 chill-sensitive species were followed.

Finally, an investigation was made into the changes in fatty-acid composition of the leaves and chloroplasts on chilling. If the degree of unsaturation were to decrease on chilling it might be expected that this would impair the reversibility of the phase change on return of the plants to the warmth. An indication of whether these changes in fatty-acid composition on chilling were detrimental to the plant was obtained by determining if these changes occurred in chill-sensitive plants which had been hardened before chilling.

MATERIALS AND METHODS

1. Growth and hardening conditions

The plants used in the experiments reported in this chapter were grown under greenhouse conditions as described in P. 24 and transferred to the growth cabinet for 2 days at 25°C before the start of the temperature treatments. The chill-sensitive species were hardened against chilling-injury as described on P. 24. The plants of Phaseolus vulgaris, Cucumis sativus, and Gossypium hirsutum (cv. Westburn 70) were 23, 30, and 56-day-old respectively at the start of the experiments. Brassica oleracea and Brassica capitata were both 10 weeks old and mature plants were used of all other species.

2. Leaf material and chloroplast isolation

The leaves were dipped in liquid nitrogen, ground in a pestle and mortar and immediately transferred to the freeze drier. The leaves were dried for 24 hours to 0.02 torr and were then ground and passed through a 500 μ sieve from which one gram was taken for analysis.

Chloroplasts were isolated by taking 50g of leaf tissue and macerating them in a Waring blendor for two minutes with 200 ml of ice cold 0.5 M sucrose solution made up in 0.01 M phosphate buffer at pH 7.4. The extract was then filtered through four layers of muslin and centrifuged at 200 G for 2 minutes at 4°C. The resulting pellet was discarded and the supernatant centrifuged at 1000 G for 10 minutes. This supernatant was discarded and the

pellet re-suspended in distilled water and re-centrifuged at 1000 G for 10 minutes. The supernatant was decanted off and the final pellet washed into a test tube with a few ml of distilled water. The preparations were freeze dried for 24 hours to 0.02 torr and the lipids extracted in the same manner as described for the leaf material below.

3. Lipid extraction

Lipids were extracted in the cold by a modification of the method of Bligh and Dyer (1959). One gram of leaf material (0.2 g of chloroplasts) was transferred to a conical flask containing 20 ml of chloroform, 40 ml of methanol and 16 ml of water. The flask was shaken for three minutes and the contents vacuum filtered in a Büchner funnel through Whatmans No. 3 filter paper. Twenty ml of chloroform was then allowed to filter through the tissue and the extract was then transferred to a separating funnel with 20 ml of water and after standing for 5 minutes the lower, green fatty-acid rich layer was taken off. The extract was washed with two further 20 ml portions of chloroform to remove any remaining traces of lipid, the chloroform extracts combined and rotary-evaporated to dryness at 30°C. To all solutions 50 µg/ml of 2, 6-ditert-butyl-p-cresol was added as anti-oxidant.

4. Preparation of the methyl esters of the fatty-acids

Methyl esters were prepared by a modification of the method of Metcalfe, Schmitz and Pelka (1966). Twenty-five ml of methanolic sodium hydroxide (0.5 N) was added to the dried

extract and refluxed for 15 minutes. Then 25 ml of 14 per cent boron trifluoride in methanol was added and refluxed for a further 15 minutes. The extract was then transferred to a separating funnel with 30 ml of saturated sodium chloride solution and 50 ml of petroleum spirit (bp 40-60°C). The funnel was shaken vigorously for 1 minute, the petrol layer separated and dried over anhydrous sodium sulphate for 30 minutes. The fatty-acid esters were extracted twice, 50 ml of petroleum being used in the second extraction. The dried petrol extract was filtered and the solution evaporated to dryness on a rotary evaporator. The methyl esters were then taken up in diethyl ether and stored at 4°C until analysis.

5. Gas-liquid chromatography

A Pye Unicam gas-liquid chromatograph model 104 equipped with a hydrogen ion flame detector was used for the analyses. The methyl esters were separated on a 1.52 m x 0.64 cm glass column with 20 per cent diethylene glycol succinate as the stationary phase. The column temperature was 190°C and the detector oven temperature 200°C. Nitrogen was used as the carrier gas at a flow rate of 35 ml per minute.

Esters were identified by comparing their retention times with those of pure methyl esters and published data obtained under similar conditions (Jamieson, 1970). The identification was further checked by silver ion thin-layer chromatography of a leaf extract of Brassica oleracea. The peak areas were

measured as peak height x retention time and the weights determined from the area produced by injecting a known weight of pure methyl heptadecanoate.

RESULTS

1. THE IDENTIFICATION OF THE METHYL ESTERS OF THE LEAF FATTY-ACIDS

The methyl esters of the fatty-acids of most plant species were identified by comparing their retention times with those of pure methyl esters and published data obtained under similar conditions. The identification of the leaf fatty-acids was further checked by silver ion thin-layer chromatography of a leaf extract of Brassica oleracea. This revealed that the gas chromatograph peak identified as 18:1 was a poorly resolved peak containing both 18:1 and 16:3. Because both these fatty-acids are unsaturated and 16:3 is generally only present in leaves in small amounts, it was not considered worthwhile performing separate analyses for these acids. Therefore, in this study, the fatty-acid identified as 18:1 may also contain traces of 16:3. A typical gas chromatograph trace of the methyl esters of the leaf fatty-acids is shown in Fig. 4 and the equivalent chain lengths of the fatty-acids is given in Table 15.

Leaves of Brassica capitata and Brassica oleracea contained approximately 44 per cent of an unknown substance which came off the column after linolenic acid and had an equivalent chain length of 22.3. Three methods were used to determine if the unknown compound was a long chain fatty-acid of Brassica oleracea leaves:-

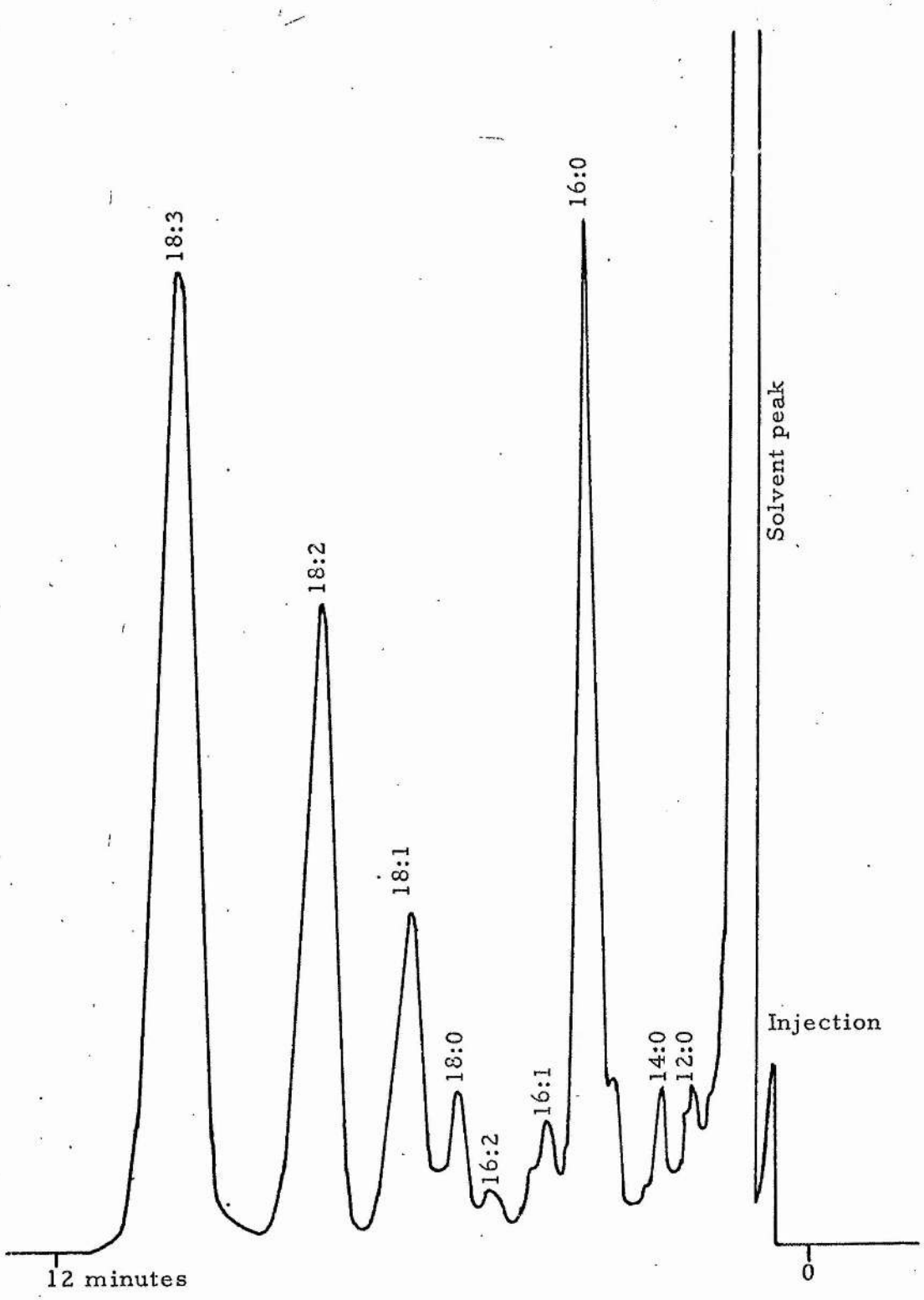


Fig. 4 . Gas Liquid Chromatography. Typical trace of the methyl esters of the leaf fatty-acids of Episcia reptans.

1) An aliquot of the prepared methyl esters of the fatty-acids of Brassica oleracea leaves was reduced with lithium aluminium hydride and the resulting alcohols converted to trimethyl silyl ethers for analysis by G. L. C. If the unknown compound is not an acid, ester or ketone, it will be unaffected by treatment with lithium aluminium hydride and appear unchanged on the G. L. C. trace.

2) To determine whether the unknown compound had any double bonds present it was isolated from the extract by silver ion thin-layer chromatography and treated with palladium/hydrogen which converts any unsaturated bonds to saturated bonds. The retention time of the compound on silver ion thin-layer chromatography was checked before and after treatment with palladium/hydrogen.

3) The unknown compound was finally analysed by mass spectroscopy.

The unknown compound was found to be unchanged after treatment with lithium aluminium hydride and palladium/hydrogen showing that it was not an ester, acid or ketone, and contained no double bonds. The mass spectrum showed the compound had a mass of 408.469192 which agrees well with the calculated mass of $C_{29}H_{60}$ which is 408.469479. This finding is in agreement with Kolattukudy (1965) who found that in the cuticular wax of cabbage

leaves the C_{29} paraffin constitutes 93 per cent of the wax hydrocarbons. A small amount (7 per cent) of the C_{29} paraffin was also present in the chloroplast preparations of Brassica capitata which may reflect that the chloroplast preparations were contaminated by leaf fragments.

The detection of a C_{29} hydrocarbon on the G. L. C. trace might lead to the suspicion that other hydrocarbons with lower chain lengths will also appear on the trace and be confused with ester peaks. However, analyses have shown that the cuticles of leaves most commonly have hydrocarbons with a chain length of C_{29} and C_{31} and contain very few hydrocarbons with chain lengths shorter than C_{27} (Hamilton and Hamilton, 1972). These hydrocarbons may only be detectable in fatty-acid analyses of those leaves which have thick cuticles and therefore large quantities of these hydrocarbons, such as Brassica oleracea and Brassica capitata. Hydrocarbons from the cuticle are not therefore considered to have interfered with the fatty-acid analyses.

The leaf cuticle consists of a meshwork of cross-esterified polymerized hydroxy fatty-acids called cutin which is embedded in a complex mixture of relatively non polar lipids commonly referred to as waxes (Kolattukudy, 1970). These hydroxy fatty-acids are not considered to have interferred with the membrane fatty-acid analyses since they generally have chain lengths greater than C_{20} (Kolattukudy, 1970) and the longest fatty-acid chain detected in the present investigation was C_{18}. In addition, methyl esters of these hydroxy fatty-acids, if formed, would

have retention times longer than C_{18} on a diethylene glycol succinate column, since they are more polar. Similarly, free fatty-acids from the cuticle are not considered to have affected the membrane fatty-acid analyses as they comprise only 9 per cent of the total cuticular waxes (Kolattukudy, 1970) and in general have chain lengths greater than C_{20}.

2. A COMPARISON OF THE LEAF AND CHLOROPLAST FATTY-ACID COMPOSITION OF CHILL-SENSITIVE AND CHILL-RESISTANT PLANTS GROWN AT 25°C

Analyses of the leaves and chloroplasts of chill-sensitive and chill-resistant plants showed no correlation between the degree of unsaturation of the fatty-acids and chill-resistance (Tables 13 and 14 respectively). This may be due to the influence of other factors on the temperature of the phase change, such as cholesterol (see P. 64).

3. CHANGES IN THE LEAF AND CHLOROPLAST FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON HARDENING AT 12°C, 95 PER CENT RH

Table 7 (Chapter 1) shows that the plants which had not been hardened before chilling suffered four times the amount of damage as the hardened plants. In spite of this reduction in injury, no increase in unsaturation of the fatty-acids was detected in either the leaves or chloroplasts of the hardened plants (Tables 15 and 16 respectively), except for a slight increase in the percentage of linoleic acid in the leaf and chloroplast analyses. This increase was not detected in Saintpaulia grandiflora.

TABLE 13. The fatty acid composition of leaves of chill sensitive and chill resistant plants grown at 25°C

(blanks denote fatty acids not detected)

Species	Fatty acid									Total
	12:0	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3	Percentage unsaturated fatty acid
Chill-sensitive										
Ipomoea batatas	0.7	0.8	17.7	3.4	-	2.1	4.6	11.9	58.8	78.7
Gossypium hirsutum	1.5	1.7	15.3	3.6	-	2.0	4.5	11.3	60.1	79.5
Episcia reptans	0.6	1.1	13.8	2.1	1.5	4.1	9.5	22.8	44.4	80.3
Saintpaulia grandiflora	0.8	1.1	14.2	2.9	1.3	3.0	10.8	23.4	42.5	80.9
Cucumis sativus	1.2	1.5	13.4	2.0	-	3.0	3.7	6.3	68.9	80.9
Phaseolus vulgaris	1.3	2.1	11.3	3.8	0.6	3.6	2.2	10.0	65.0	81.6
Coleus blumei	0.5	1.0	12.3	2.0	0.1	2.5	2.8	17.0	61.8	83.7
Chill-resistant										
Saxifrage umbrosa	1.7	3.9	16.3	-	-	-	2.5	31.5	44.2	78.2
Brassica capitata	2.3	2.6	12.7	4.9	3.1	4.2	12.8	13.7	43.7	78.2
Brassica oleracea	0.8	1.6	13.1	3.2	1.6	1.7	13.5	17.6	46.8	82.7
Beta vulgaris	0.8	0.8	13.4	2.3	-	1.1	5.2	14.9	61.5	83.9
Brassica rapa	0.9	0.9	12.4	1.9	-	1.8	14.8	9.4	57.9	84.0
Ligustrum ovalifolium	0.7	0.7	13.0	2.1	0.6	1.2	6.0	9.0	66.7	84.4

Table 14. The percentage fatty-acid composition of the chloroplasts of chill-sensitive and chill-resistant plants grown at 25°C

(blanks denote fatty-acids not detected)

Species	Fatty-acid									Total percentage unsaturated fatty-acid
	*12:0	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3	
Chill-sensitive										
Gossypium hirsutum	1.8	3.1	18.8	-	-	2.1	3.5	10.0	60.7	74.2
Cucumis sativus	3.3	3.7	23.6	5.9	2.0	4.1	5.3	3.7	48.4	65.3
Phaseolus vulgaris	2.1	2.6	18.7	5.5	1.0	2.0	1.3	4.2	62.6	74.6
Zea mays	3.3	3.6	21.9	4.8	1.4	2.1	1.5	3.8	57.6	69.1
Chill-resistant										
Brassica capitata	1.9	2.5	17.8	2.9	2.6	1.4	15.6	10.1	45.2	76.4
Beta vulgaris	2.3	2.6	15.3	-	-	1.5	4.8	6.0	67.5	78.3

*Ratio shown is the number of carbon atoms to the number of double bonds in the molecule.

TABLE 15. Changes in fatty acid composition of the leaves of chill sensitive plants on hardening at 12°C, 95 per cent RH

Fatty Acid	Equivalent Chain Length	Gossypium hirsutum		Phaseolus vulgaris		Saintpaulia grandiflora		Cucumis sativus	
		Control	Hardened	Control	Hardened	Control	Hardened	Control	Hardened
12:0	12.0	1.5	1.7	-	-	0.8	0.8	0.6	0.6
14:0	14.0	1.7	2.0	0.6	0.6	1.3	1.2	1.7	1.0
16:0	16.0	15.3	15.3	12.2	11.9	14.9	14.0	14.5	14.4
16:1	16.7	3.6	2.7	2.7	2.4	3.0	3.1	2.3	2.3
16:2	17.5	-	-	-	-	1.2	1.3	-	-
18:0	18.0	2.0	2.5	2.3	1.9	3.0	3.2	2.5	3.0
18:1	18.4	4.5	4.5	2.0	1.9	10.9	9.8	6.2	6.2
18:2	19.2	11.3	14.6	9.8	12.5	23.2	23.5	6.6	8.0
18:3	20.2	60.1	56.7	70.4	68.8	41.7	43.1	65.6	64.5
Total percent unsaturated fatty-acid		79.5	78.5	84.9	85.6	80.0	80.8	80.7	81.0

Table 16. Changes in the percentage fatty-acid composition of the chloroplasts of chill-sensitive and chill-resistant plants on hardening at 12°C, 95 per cent RH

Fatty-acid	Gossypium hirsutum		Phaseolus vulgaris		Beta vulgaris		Brassica capitata	
	Control	Hardened	Control	Hardened	Control	Hardened	Control	Hardened
12:0	1.8	1.9	2.9	3.0	2.3	2.4	1.9	1.8
14:0	3.1	2.7	2.9	3.2	2.6	3.0	2.5	2.3
16:0	18.8	18.4	17.6	17.8	15.4	15.3	17.8	18.1
16:1	-	-	6.1	6.1	-	-	2.9	2.0
16:2	-	-	-	-	-	-	2.6	2.8
18:0	2.1	2.0	2.0	3.0	1.5	1.6	1.4	1.7
18:1	3.5	3.8	0.7	2.3	4.8	4.9	15.6	14.9
18:2	9.9	12.6	2.5	4.6	6.0	6.2	10.1	10.6
18:3	60.8	58.6	65.3	60.0	67.4	66.6	45.2	45.8
Total per cent unsaturated fatty-acid	74.2	75.0	74.6	73.0	78.2	77.7	76.4	76.1

Hardening the chill-sensitive species resulted in a decrease in the total weight of fatty-acid of approximately 25 per cent in the leaves and chloroplasts (Tables 17 and 18 respectively). These decreases may be due to the oxidation of lipids as a source of energy. In the chill-resistant species the weight of fatty-acid in the leaves and chloroplasts showed little change due to the hardening treatment.

4. CHANGES IN THE LEAF FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON CHILLING AT 5°C, 85 PER CENT RH

When chill-sensitive species were chilled there was a decrease in the percentage of linolenic acid and total weight of fatty-acids (Figs. 5 and 6). The decrease in the percentage of linolenic acid was particularly rapid in the very chill-sensitive plants of Cucumis sativus and Saintpaulia grandiflora and slower in the less chill-sensitive Coleus blumei. The decreases in the percentage of linolenic acid resulted in a decrease in the total percentage of unsaturated fatty-acid in the leaf.

In chill-resistant species there was no change in either the percentage of linolenic acid or total weight of fatty-acids on chilling (Figs. 5 and 6).

Table 17. Changes in total weight of leaf fatty-acids of chill-sensitive and chill-resistant plants on hardening at 12°C, 95 per cent RH. (Expressed as mg of fatty-acid per gram of lyophilized leaf)

	Species	Control	Hardened
Chill-sensitive	Gossypium hirsutum (Westburn)	19.7	14.3
	Phaseolus vulgaris	25.1	18.5
	Cucumis sativus	27.3	26.6
Chill-resistant	Brassica capitata	8.1	7.1
	Beta vulgaris	13.6	16.3

Table 18. Changes in the total weight of chloroplast fatty-acids of chill-sensitive and chill-resistant plants on hardening

(expressed as mg of fatty-acid per gram of lyophilized chloroplast)

Species		Control	Hardened
Chill-sensitive	Gossypium hirsutum (Westburn)	61.3	46.8
	Phaseolus vulgaris	63.0	50.5
	Cucumis sativus	77.1	68.3
Chill-resistant	Brassica capitata	61.4	60.9
	Beta vulgaris	78.0	76.0

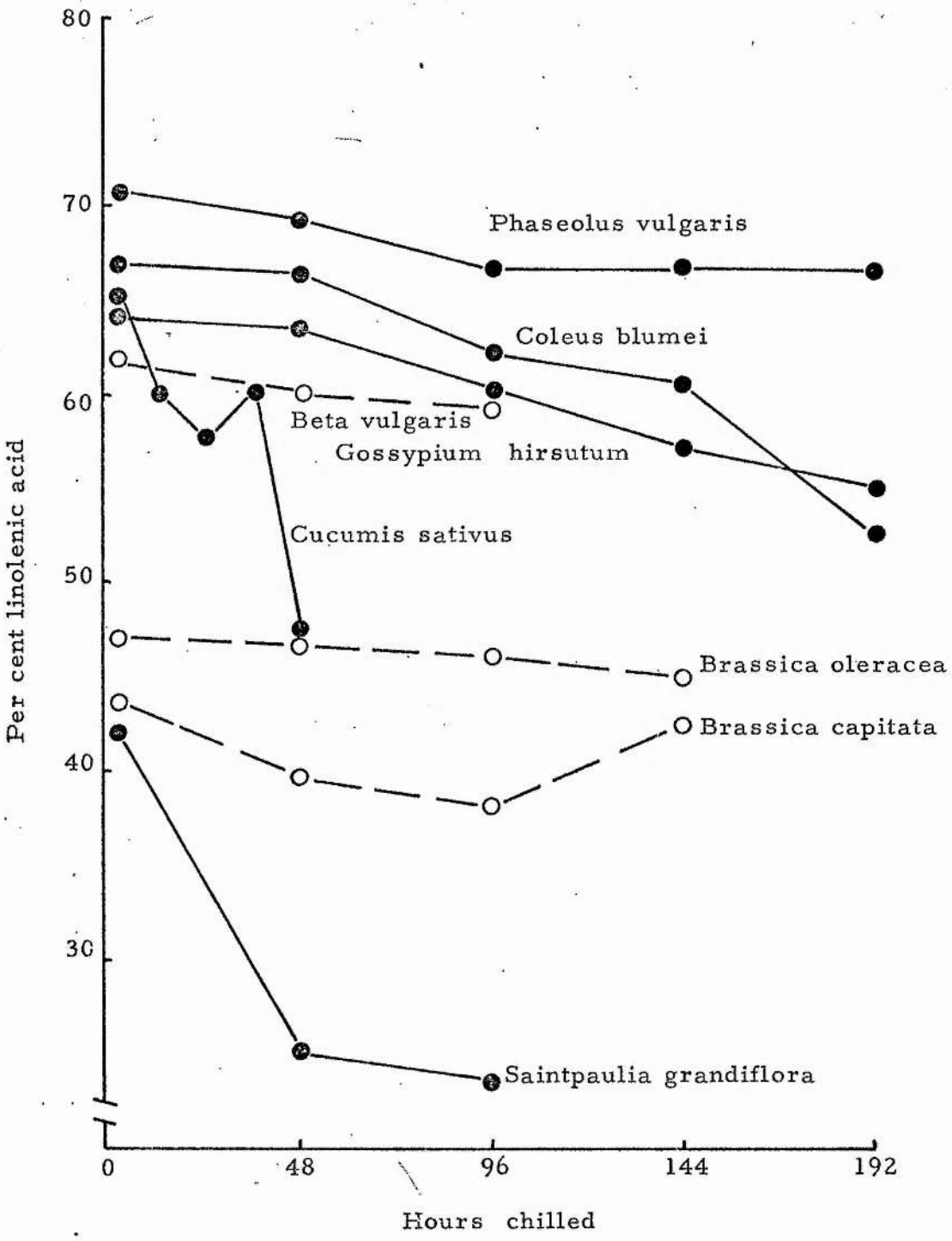


Fig. 5. Time course of changes in percentage of linolenic acid in leaves on chilling at 5°C, 85 per cent RH. Continuous lines indicate chill-sensitive plants and broken lines chill-resistant plants.

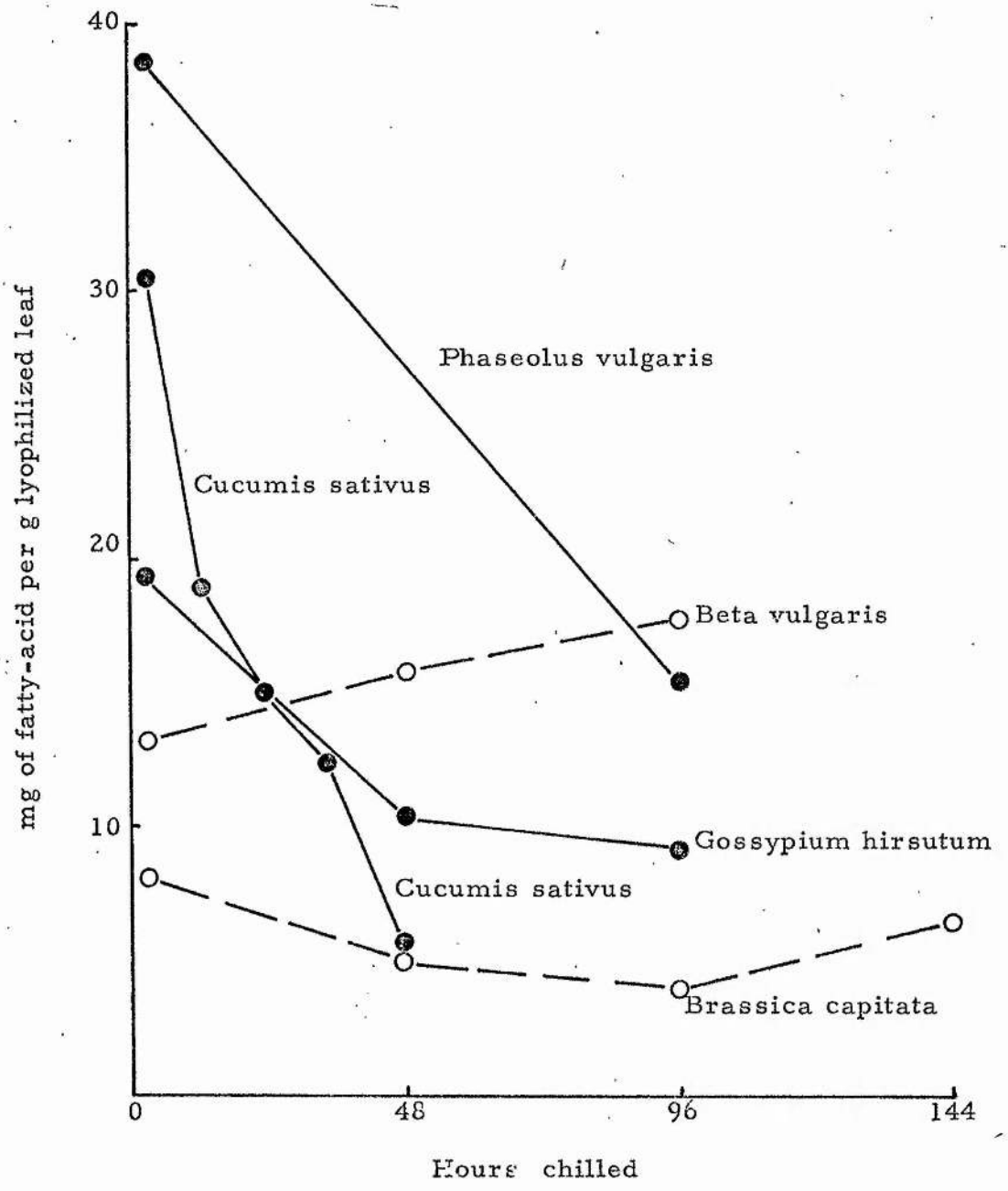


Fig. 6. Time course of changes in weight of fatty-acid in leaves on chilling at 5°C, 85 per cent RH. Continuous lines indicate chill-sensitive plants and broken lines chill-resistant plants.

5. CHANGES IN THE CHLOROPLAST FATTY-ACID COMPOSITIONS OF CHILL-SENSITIVE AND CHILL-RESISTANT SPECIES ON CHILLING AT 5°C, 85 PER CENT RH

Chilling increased the percentage of linolenic acid and total weight of fatty-acid in the chloroplasts of chill-sensitive leaves (Figs. 7 and 8 respectively). Because the percentage of linolenic acid decreased in the whole leaf analysis on chilling, the decrease in the plasmalemma and other membrane systems outside the chloroplast must be particularly large as it is generally considered that the chloroplast contains the majority of the cellular lipids and fatty-acids. It is therefore thought that the decrease in the percentage of linolenic acid in the leaf on chilling may play an important role in determining the extent of damage to the plant.

In chill-resistant plants the percentage of linolenic acid in the chloroplast either remained constant on chilling (Beta vulgaris) or decreased (Brassica capitata). The total weight of fatty-acids in the chloroplasts decreased on chilling in both chill-resistant species. Therefore the changes in fatty-acid composition of the chloroplasts of chill-resistant plants on chilling are different to those which occur in chill-sensitive plants.

6. CHANGES IN THE LEAF FATTY-ACID COMPOSITIONS OF HARDENED LEAVES DURING CHILLING AT 5°C, 85 PER CENT RH

Further evidence that the decrease in the percentage of linolenic acid on chilling chill-sensitive leaves was detrimental to the plant

was obtained by following the changes in the fatty-acid composition of plants that had been hardened prior to chilling. Figure 9 shows that in Cucumis sativus and Saintpaulia grandiflora the decrease in the percentage of linolenic acid was reduced if the plants had been hardened before chilling.

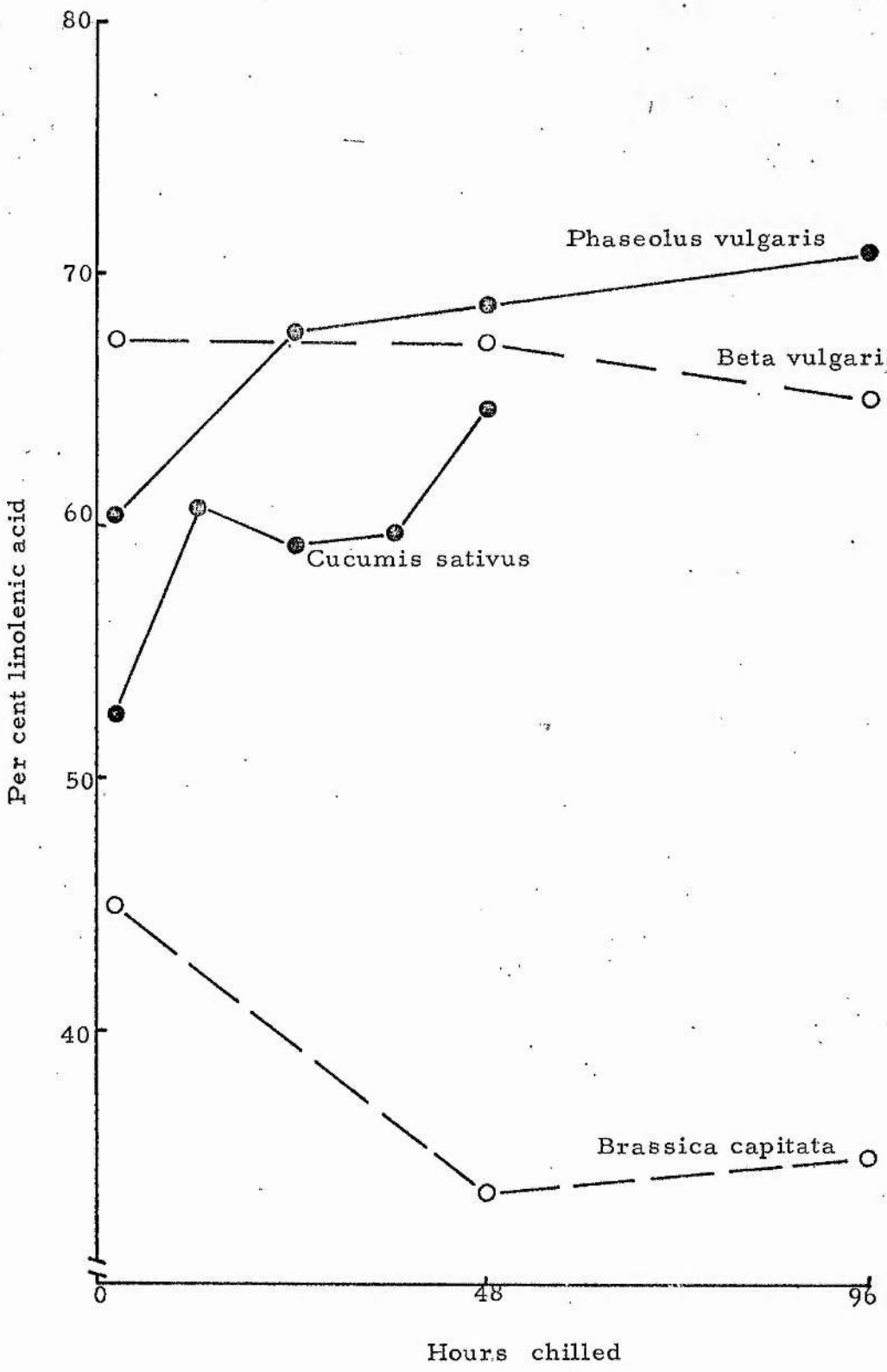


Fig. 7. Time course of changes in percentage of linolenic acid in chloroplasts on chilling at 5°C, 85 per cent RH. Continuous lines indicate chill-sensitive plants and broken lines chill-resistant plants.

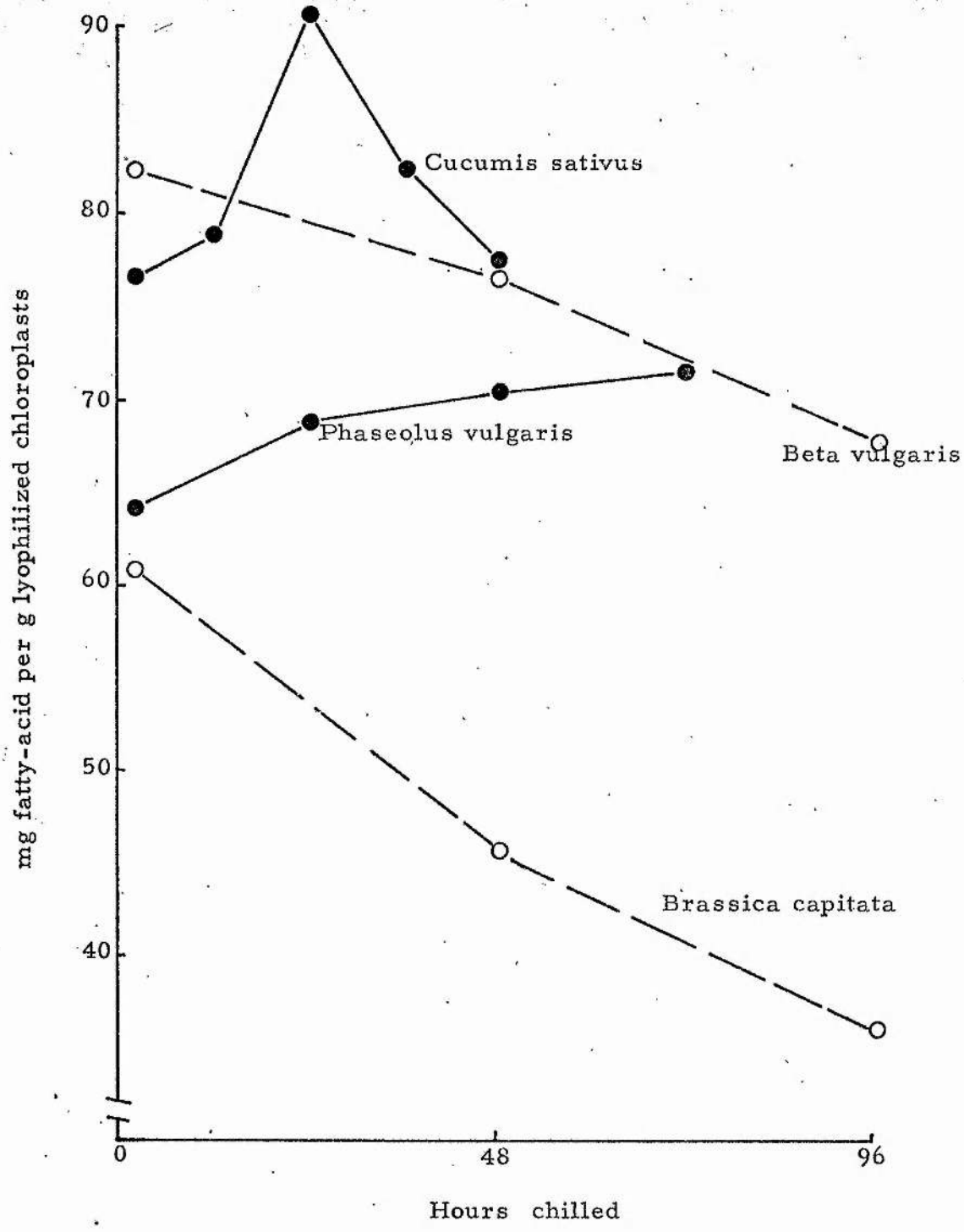


Fig. 8. Time course of changes in weight of fatty-acid in chloroplasts on chilling at 5°C, 85 per cent RH. Continuous lines indicate chill-sensitive plants and broken lines chill-resistant plants.

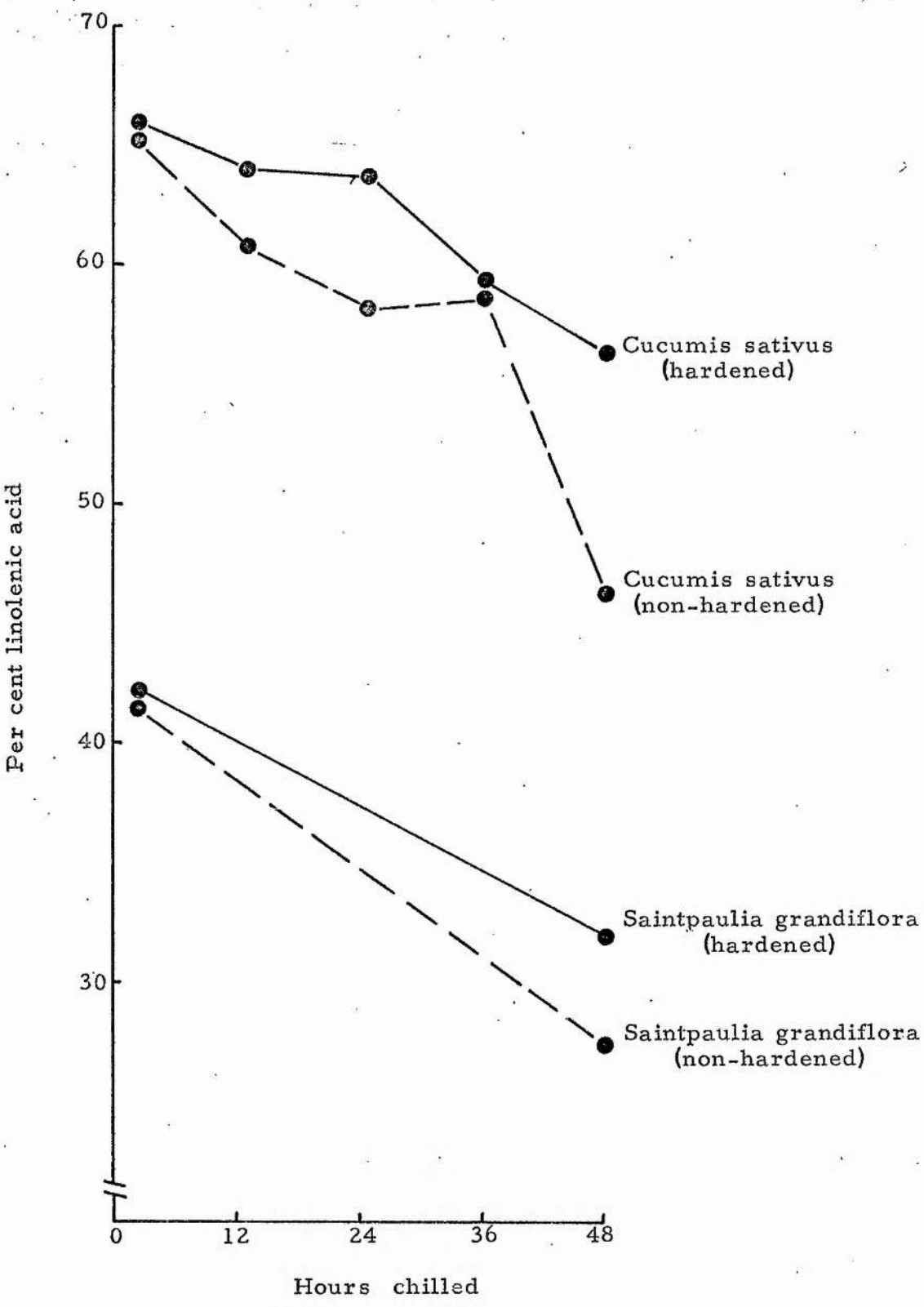


Fig. 9. Time course of changes in percentage of linolenic acid in the leaves of hardened and non-hardened chill-sensitive plants on chilling at 5°C, 85 per cent RH. Continuous lines indicate plants which had been hardened for 4 days at 12°C, 95 per cent RH, before chilling, and broken lines plants which remained at 25°C before chilling.

DISCUSSION

The results of this chapter cast further doubt on the importance of the degree of unsaturation of the fatty-acids as the dominant factor determining the critical temperature at which the phase change occurs in plant membranes. When plants were grown at 25°C under similar lighting conditions, leaves and chloroplasts of chill-resistant species did not contain more unsaturated fatty-acid than chill-sensitive species. This result may be due to changes in fatty-acid composition of the leaves (P. 182) and chloroplasts (Newman, 1962) with increase in physiological age, since the variation in the fatty-acid composition with increase in age could not be eliminated in this study due to problems of growing many different species with leaves of identical ages.

There was no increase in unsaturation or weight of fatty-acid on hardening chill-sensitive species, except for a slight increase in the percentage of linoleic acid. It appears that the increases in unsaturation and weight of fatty-acid that occur on hardening plants to sub-zero temperatures (P. 8) do not occur on hardening chill-sensitive plants to chilling temperatures. An explanation of hardening in chill-sensitive species must therefore be sought elsewhere. Unfortunately little is known about the changes in the level of cholesterol on hardening plant tissues. Davis and Finkner (1972) detected no increase in the level of cholesterol in wheat shoots when the temperature was lowered from 10 to 1°C. It is possible that hardening against chilling-injury results in an increase in the degree of unsaturation of a specific membrane system or lipid which was not detected by analysing total leaf fatty-acid.

The effects of chilling on the reversibility of the phase change

The decreases in the percentage of linolenic acid and total weight of fatty-acid on chilling leaves of chill-sensitive species (Figs. 5 and 6) may alter the temperature at which the phase change reverses on return to the warmth. The transition temperature depends on the degree of unsaturation of the fatty-acids (Lyons and Asmundson, 1965). If the degree of unsaturation decreases during chilling then the phase change will only reverse at a much higher temperature, perhaps too high for the growth of the plant. It is possible that the decrease in linolenic acid in specific membrane systems outside the chloroplast may be faster than is indicated by the whole leaf analyses, as shown by the increase in the percentage of linolenic acid in the chloroplasts on chilling. The decreases in the percentage of linolenic acid and weight of fatty-acids in the chilled leaves of chill-sensitive plants indicates membrane breakdown, and this may explain the progressive nature of chilling-injury with increasing length of exposure to chilling temperatures.

The decrease in the percentage of linolenic acid in the chilled leaves of chill-sensitive plants may be due to a decrease of this fatty-acid in both the neutral and polar lipid fractions. Alternatively, the decrease may be equally well explained by the fact that the polar lipids, MGDG and DGDG, which are predominantly esterified with linolenic acid, are particularly rapidly oxidised as a source of energy, although there is very little evidence that these lipids are used as an energy reserve in leaves. The rapid decreases in linolenic acid on chilling Cucumis sativus and Saintpaulia grandiflora

suggest that this fatty-acid may undergo autoxidation since unsaturated fatty-acids are more susceptible to autoxidation than saturated fatty-acids (Hitchcock and Nichols, 1971). Whether the linolenic acid associated with all the lipids is equally susceptible to autoxidation is not known, although it would be expected that those lipids with a high percentage of linolenic acid such as MGDG would be more susceptible.

Autoxidation

The mechanism of the primary process of autoxidation is the formation of a hydroperoxide by a free radical chain reaction and is probably highly injurious to the cells (Christopherson, 1969). The free radicals produced in peroxidising lipid-protein reaction systems result in proteins and enzymes losing their solubility and the destruction of their constituent amino acids (Roubal and Tappel, 1966). Furthermore, the primary reaction of autoxidation may be catalysed by lipoxygenases which have been shown to be widely distributed in plant leaves (Holden, 1970). The results of Hatefi and Hanstein (1970) further support the theory that autoxidation may occur on chilling. They reported that a potentially destructive process such as lipid autoxidation is built into the enzymic machinery of mitochondria and that when mitochondria are under stress by the overloading or exhaustion of substrates, lipid oxidation can take place. When photosynthesis is inhibited by chilling, illumination may result in a sensitized photo-oxidation which occurs at a much faster rate (Cobern, Hobbs, Lucas and Mackenzie, 1966; Lomagin and Antropova, 1966). The prevention of photo-oxidation

by chilling plants in the dark may explain the reduction in the degree of damage to these plants (P. 45). Hardening may prevent autoxidation by increasing the degree of unsaturation of minor membrane components not detected here, thus lowering the temperature of the phase change, or by reducing the activity of lipoxygenase on chilling. In addition, hardening may reduce autoxidation by increasing the activity of hydroperoxide isomerases which have been shown to be widely distributed in plant tissues (Zimmerman, Vick and Borg, 1974). An increase in the ratio of NADPH:NADP also occurs on hardening (Kuraishi, et al., 1968) which may stop lipid peroxidation by scavenging the oxygen in the cell.

The effects of chilling on chloroplast fatty-acid composition

In chill-sensitive species the percentage of linolenic acid and total weight of fatty-acid in the chloroplasts increased on chilling and decreased in the chill-resistant species. These different responses to chilling may be due to changes in the amount of osmiophilic globules in the chloroplast since they have been shown to have a galactolipid composition (Gibbs, 1971). During the degeneration of senescent or damaged chloroplasts the number of globules increases which may be the cause of the increase in linolenic acid and weight of fatty-acid in chloroplasts on chilling chill-sensitive species. The decrease in the percentage of linolenic acid and total weight of fatty-acid in the chloroplasts of chill-resistant species on chilling may be due to the utilization of the osmiophilic globules for membrane synthesis outside the chloroplasts. It has been shown that these globules may serve as reservoirs of lipid for membrane development, and hardening of chill-resistant species against frost-damage is thought to result in increased membrane synthesis (P. 175).

Because the fatty-acid composition of the chloroplast may fluctuate according to light intensity and the amount of osmiophilic globules present, the fatty-acid composition of these organelles may bear little relation to their chill-sensitivity. Nichols, Stubbs and James (1967) have shown that light increases the degree of unsaturation of the glycolipids and, to a lesser extent, the phospholipids of castor leaf chloroplasts. This situation is analogous to that which occurs on the dietary modification of rat liver mitochondria (Williams, et al., 1972) which resulted in large increases in unsaturation of the membrane phospholipids but did not alter the transition temperature.

In conclusion, the results suggest that the degree of unsaturation of the total membrane fatty-acids does not have a dominant role in determining the temperature at which the phase change occurs in chill-sensitive plants. It is possible that the transition temperature is dependent on the degree of unsaturation of specific lipid components such as the phospholipids and not the overall fatty-acid composition of the lipid matrix. Hardening may therefore produce increases in unsaturation of specific lipids which are only minor components of membranes in terms of total fatty-acid composition, as indicated by the slight increase in linoleic acid on hardening. The changes in the fatty-acid composition of leaf phospholipids and glycolipids in relation to hardening and chilling-injury are reported in the following chapter.

SUMMARY

The leaves and chloroplasts of chill-resistant plants grown at 25°C did not contain more unsaturated fatty-acid than chill-sensitive plants. Furthermore, when chill-sensitive plants were hardened there was no increase in the amount of unsaturated fatty-acid in the leaves or chloroplasts, except for a slight increase in linoleic acid. Hardening did not increase the weight of fatty-acid in the leaves or chloroplasts of either the chill-sensitive or chill-resistant plants. Therefore, changes which occur on hardening plants to sub-zero temperatures did not occur on hardening chill-sensitive species. These results cast further doubts on the importance of the total degree of unsaturation of the membrane fatty-acids in determining the critical temperature at which the phase change occurs in the lipid layer of the membranes.

When chill-sensitive leaves are chilled the percentage of linolenic acid and total weight of fatty-acid decreases rapidly but remains constant in chill-resistant species. In the chloroplasts of chill-sensitive species the percentage of linolenic acid and weight of fatty-acid increases on chilling indicating that the decrease in linolenic acid in membrane systems outside the chloroplast may be larger than that indicated by the fatty-acid analysis of the whole leaf. Hardening may reduce the degree of damage to the plant by slowing down detrimental processes such as the decrease in the percentage of linolenic acid which occurs in the leaves of chill-sensitive species on chilling.

CHAPTER FOUR

THE ACCLIMATIZATION OF PLANTS TO CHILLING TEMPERATURES IN RELATION TO THE FATTY-ACID COMPOSITION OF LEAF POLAR LIPIDS

INTRODUCTION

In the previous chapter a comparison of the fatty-acid compositions of the leaves and chloroplasts of 13 species of chill-sensitive and chill-resistant plants grown at 25°C showed no differences which could be related to susceptibility to chilling-injury. Furthermore, no increase in the degree of unsaturation of the total leaf and chloroplast fatty-acids could be detected during the hardening of 4 chill-sensitive species at 12°C, except for a small increase in linoleic acid (Chapter 3). Because the fatty-acids associated with the phospho- and glycolipids comprise only 22 and 38 per cent respectively of the total leaf fatty-acid, it seemed possible that changes in unsaturation of the polar lipids on hardening might not have been detected by analysing total leaf fatty-acid due to the very small changes involved.

Three investigations have suggested that the temperature at which the phase change occurs may be related to the degree of unsaturation of the phospholipids and not the overall fatty-acid composition of the lipid layer. Firstly, Lyons, et al., (1964) found that the fatty-acids of mitochondria isolated from chill-resistant fruits were more unsaturated than those of chill-sensitive fruits. Because 54 per cent of the total lipid of plant mitochondria is phospholipid (P. 11), it appears that

their results were due to a greater degree of unsaturation of the phospholipids in chill-resistant species. Secondly, Shneyour, et al., (1973) have shown that in chloroplasts of chill-sensitive species a phase change occurred at 12°C, even though the overall degree of unsaturation is large in these organelles due to high levels of MGDG and DGDG and therefore a phase change would not be expected until well below 0°C. Thirdly, Steim (1972) has shown that changes in the active transport of membranes of Escherichia coli take place at discreet temperatures, anywhere within the temperature range circumscribed by the bulk phase change as detected by calorimetry. These changes in active transport are considered to be due to lipid phase changes in the immediate neighbourhood of the enzyme protein. Therefore, it was decided to investigate the fatty-acid compositions of the polar lipids of leaves in relation to their chill-sensitivity.

Although total fatty-acid analyses of the mitochondria isolated from chill-sensitive and chill-resistant fruits have been made (Lyons, et al., 1964), no analyses have been performed to determine the fatty-acid composition of individual membrane lipid components in plant leaves grown at 25°C in relation to their susceptibility to chilling-injury. Because it is not possible to isolate mitochondria from leaves due to chloroplast contamination, it was considered that analysis of the fatty-acid composition of the polar lipids of chill-sensitive and resistant leaves grown at 25°C would reveal

any differences which could be related to the chill-sensitivity of the species. However, it is not possible to determine from which membrane system the phospholipid originated since all membranes contain significant amounts of every phospholipid present in the leaf (Table 19).

Analyses of the polar lipids of leaves in relation to hardening and chilling have so far been confined to the measurement of weight of phospholipid as the method of analysis has, until recently, involved the destruction of the fatty-acids (Wright and Simon, 1973; Guinn, 1971). Wright and Simon (1973) found that the weight of all phospholipids decreased rapidly after chilling cucumber leaves for 2 days and Guinn (1971) showed that the level of lipid soluble phosphate decreased on hardening Gossypium hirsutum. Gas-liquid chromatography of the methyl esters of the fatty-acids with added internal standard now enables the determination of both weight and fatty-acid composition of the lipid from a single sample (Christie, Noble and Moore, 1970). The esterification of the fatty-acids can be performed directly on the thin-layer adsorbent used in the separation of the lipids by thin-layer chromatography (Christie, 1972). This chapter reports the changes in weight and fatty-acid composition of the polar lipids of two chill-sensitive species, Phaseolus vulgaris and Gossypium hirsutum and one chill-resistant species, Hordeum vulgare, on exposure to a hardening temperature of 12°C, 95 per cent RH and a chilling temperature of 5°C, 85 per cent RH. In addition, the changes in the fatty-acid composition of the polar lipids of Phaseolus vulgaris during 4 days growth at 5°C, 100 per cent RH and 12°C, 100 per cent RH, were

followed. These treatments at 100 per cent RH, by enclosure in polythene bags, were not effective against subsequent chilling at 5°C, 85 per cent RH (P. 35). The changes in the fatty-acid composition of the polar lipids of Episcia reptans during the ineffective hardening at 15°C, 95 per cent RH, were also investigated.

Finally, a comparison was made of the changes in weight and fatty-acid composition of the polar lipids of chill-sensitive and resistant plants with increase in physiological age over a 4-day period at 25°C because it had been discovered that older leaves of chill-sensitive species were more susceptible to chilling-injury (Chapter 1). These experiments provided further controls for the changes which occurred during hardening. Research on the changes in the fatty-acid composition of alfalfa leaves during maturation has shown that the percentage of linolenic acid decreases from 30 to 10 per cent of the total weight of phospholipid fatty-acid over 200 days (Klopfenstein and Shigley, 1967). During this period palmitic acid increased by 10 per cent and linoleic acid by 4 per cent. Similar results were obtained by Hawke (1963) who analysed the total leaf fatty-acid composition of rye grass and for the chloroplasts of bush bean nodes by Newman (1962).

Table 19. Distribution of polar lipids in the various cell fractions isolated from tobacco leaves (Data from Ongun, et al., 1968). Values are expressed as a percentage of the total weight of the lipid present.

Lipids	Chloroplast 1,000 G	Mitochondrial fraction 15,000 G	Supernatant
Glycolipids:			
MGDG	83	8	9
DGDG	88	3	9
SL	76	14	9
Total glycolipid distribution	82	8	9
Phospholipids:			
PC	27	12	59
PE	38	19	42
PI	13	56	30
PG	74	13	12
Total phospholipid distribution	38	25	36

MATERIALS AND METHODS

1. Growth and hardening conditions

The seeds of chill-sensitive and resistant plants were sown in 7 cms pots containing John Innes potting compost No. 2 and germinated in the hot-house under mist spray at approximately 25°C before transfer to the growth cabinet at 25°C and 95 per cent relative humidity. The seeds of Phaseolus vulgaris (chill-sensitive) were germinated for 4 days in the hot-house until the seed appeared above the surface of the soil. They were then transferred to the growth cabinet for 3 days and the first two leaves used for the analyses. In the case of Gossypium hirsutum cv. Parrot (chill-sensitive) the third leaf was used for the analyses as the first two leaves were of poor quality. The plants were grown for 13 days under hot-house conditions until the third leaf just became visible and then transferred to the growth cabinet for 5 days. Hordeum vulgare (chill-resistant) was sown at a density of 10 seeds per pot and germinated in the hot-house for 3 days until the shoot appeared above the surface of the soil. The plants were then grown in the growth cabinet for 4 days and the first leaf used for the analyses. Due to the slow growth rate of Episcia reptans the leaves of this species could not be grown entirely under growth-cabinet conditions. Therefore the young leaves of 8-week-old cuttings which had been grown under mist spray in the hot-house were used for the experiments. Because Episcia reptans is chill-injured at 12°C and does not harden against chilling at 8°C by 4 days' growth at 15° C, young leaves

of this species which had been grown for 4 days at 15°C were analysed to determine whether the changes in the fatty-acid composition over this period were similar to those which occurred during the effective hardening of Gossypium hirsutum and Phaseolus vulgaris.

After the requisite period for the expansion of the leaves of Phaseolus vulgaris, Gossypium hirsutum and Hordeum vulgare at 25°C, the plants were given the following temperature treatments:-

1) Hardened for 4 days at 12°C, 95 per cent RH, and then chilled at 5°C, 85 per cent RH.
2) Chilled directly at 5°C, 85 per cent RH having received no hardening treatment.
3) Plants of Phaseolus vulgaris were grown for 4 days at 12°C, 100 per cent RH, or 5°C, 100 per cent RH, by enclosure in polythene bags. These treatments were not effective in preventing injury on subsequent chilling at 5°C, 85 per cent RH (P. 35).
4) Grown for a further 4 days at 25°C as controls for the hardening treatments.

It was considered that the temperature treatments described above may have altered the susceptibility of chill-resistant Hordeum vulgare to frost-injury. Growth of this species for 1 day at 5°C or 4 days at 12°C might increase the resistance of the plants to sub-zero temperatures, and the growth of the controls for a further 4 days at 25°C may lower the resistance of the plants to

frost-injury. To test these hypotheses, plants of Hordeum vulgare were frozen at -4°C for 8, 13 and 24 hours. Unfortunately the plants had to be frozen in the dark as the temperature of the growth cabinet could not be lowered to -4°C with the lights on. Resistance to freezing was estimated as the number of plants alive after 72 hours recovery at 15 to 20°C, 70 per cent RH.

2. Lipid extraction

The leaves were immersed in liquid nitrogen, ground in a pestle and mortar and immediately freeze-dried for 24 hours to 0.02 mm Hg. The dried material was then ground and passed through a 500 µm sieve from which 1 g was taken for analysis. Samples were stored over anhydrous calcium chloride in a vacuum desiccator at -20°C.

Lipids were extracted by a modification of the method of Bligh and Dyer (see P. 69) and the extract was rotary evaporated to dryness at 30°C. The flask was then cooled in ice and 2 ml of ice-cold chloroform was used to dissolve the extract.

3. Thin-layer chromatography

The thin-layer plates were cleaned by soaking in concentrated sulphuric acid and then washing several times in hot water before finally rinsing in distilled water. The polar lipids were separated by two-dimensional thin-layer chromatography on glass plates (20 cms x 20 cms) coated with a 1 mm thick layer of silica gel G (Merck). The plates were allowed to dry overnight after spreading

the silica gel and then dried for 30 minutes at 110°C. After cooling for 10 minutes in a desiccator cabinet an aliquot (0.1 ml) of the extract was applied as a spot to each plate and allowed to dry for 5 minutes. The plates were developed chloroform; methanol; ammonium hydroxide (65:30:4) for the first direction and chloroform; methanol; acetic acid; water (170:25:25:6) in the second direction (Nicholls and James, 1964). To all solutions 50 µg/ml of 2, 6-di-tert-butyl-p-cresol was added as anti-oxidant. The chromatography tanks were lined with filter paper, the lids sealed with silicone grease and a heavy weight, and allowed to equilibrate for 12 hours before use. The plates were developed for one hour in each direction and dried for 15 minutes between developments over a slow stream of nitrogen gas at 25°C.

4. Identification of lipids

The lipid spots were detected with iodine vapour and initially identified by comparison with published results obtained under similar conditions (Ongun, Thomson and Mudd, 1968). The phospholipids were identified by spraying the plates with molybdenum reagent (Allen and Good, 1970) and by chromatography with standards except for phosphatidyl glycerol which was not available. Standards were supplied by the Sigma Chemical Co. Ltd. Five phospholipids were identified:-

Lipid	Abbreviation	Standard used in identification
Phosphatidyl choline	PC	DL -α- Lecithin, synthetic
Phosphatidyl ethanolamine	PE	DL -α- Cephalin, synthetic (practical grade)

Lipid	Abbreviation	Standard used in identification
Phosphatidyl inositol	PI	Brain Extract, type I
Phosphatidyl glycerol	PG	Not available
Phosphatidic acid	PA	Phosphatidic acid, sodium salt. (Lyophilized from egg lecithin)

Glycolipids were identified by spraying the plates with α-napthol reagent (Allen and Good, 1970). These were:-

MGDG - Monogalactosyl diglyceride
DGDG - Digalactosyl diglyceride
SL - Sulpholipid

Further confirmation of correct identification was subsequently obtained from the fatty-acid analyses of the lipids.

A further two lipids (spots 10 and 11 in Fig. 10) were detected when the plates were sprayed with 0.1 per cent dichlorofluorescein. These spots from 4 plates were analysed by gas-liquid chromatography but only a trace of fatty-acid was detected. Spot 11 turned blue with molybdenum reagent and is therefore thought to be a phospholipid.

5. Preparation of the methyl esters of the fatty-acids

The developed plates were sprayed lightly with 0.1 per cent 2, 7 -dichlorofluorescein in ethanol and the lipid spots viewed under ultra-violet light and marked. The spots from 4 plates were scraped into 30 ml glass stoppered test tubes and the methyl esters of the fatty-acids prepared on the thin-layer adsorbent according to Christie (1972). An internal standard of 1 ml of methyl heptadecanoate

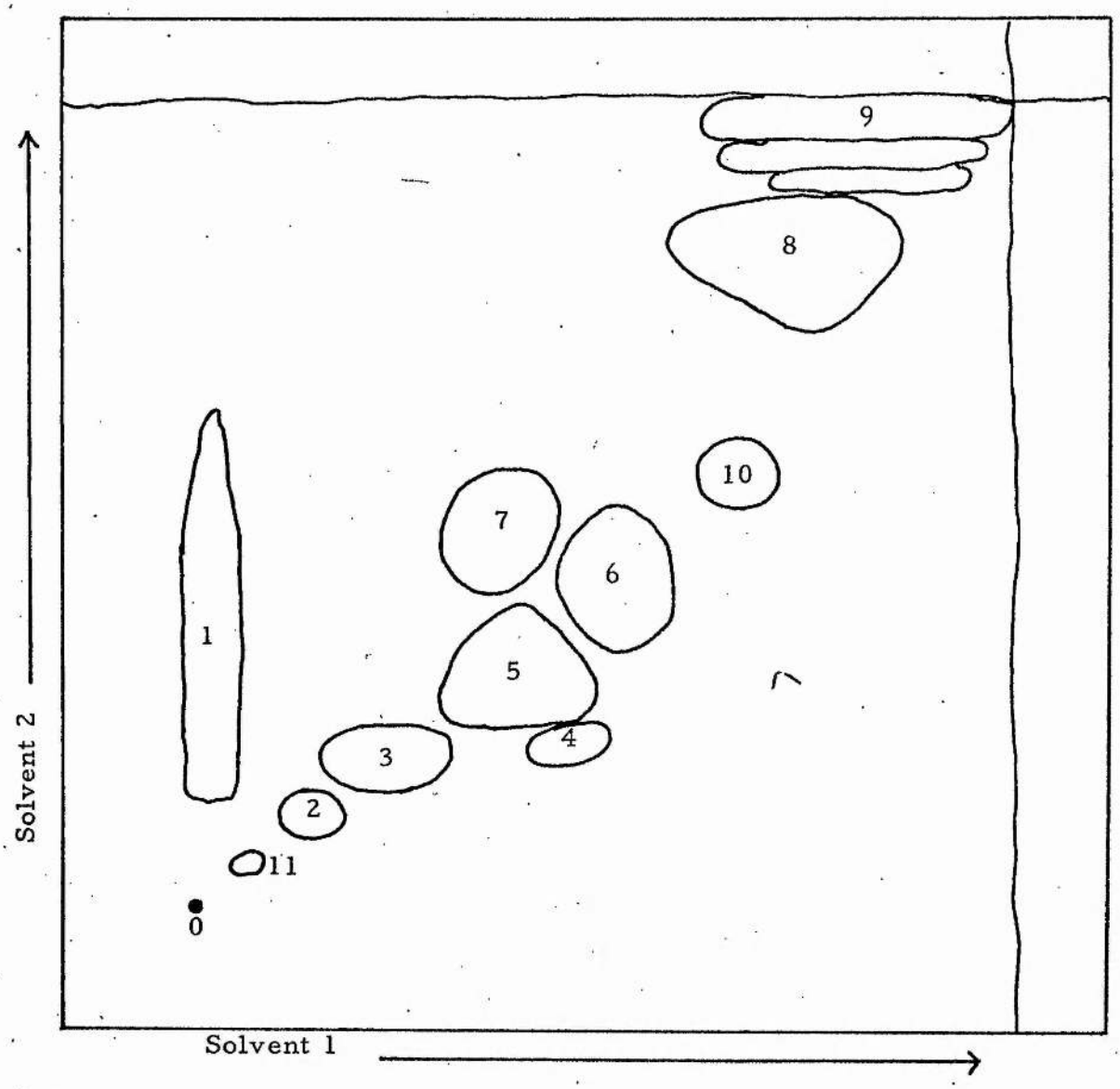


Fig. 10. Two-dimensional thin-layer chromatography plate ($x\frac{3}{4}$) of the separation of phospholipids and glycolipids by the method of Nicholls and James, 1964. Identification of components: 1, phosphatidic acid; 2, phosphatidyl inositol; 3, phosphatidyl choline; 4, sulpholipid; 5, digalactosyl diglyceride; 6, phosphatidyl glycerol; 7, phosphatidyl ethanolamine; 8, monogalactocyl diglyceride; 9, pigments and neutral lipids; 10, 11, unidentified lipids; 0, origin.

in methanol was added to the tubes followed by 2 ml of 2 N sodium methoxide (the sodium methoxide was prepared by dissolving pure sodium metal in specially dried methanol). The tubes were then stoppered, shaken vigorously for 2 minutes and then heated at 50°C in a water bath for 15 minutes. After cooling, the sodium methoxide solutions were acidified with 0.6 ml of glacial acetic acid, 5 ml of distilled water was then added and the esters extracted into di-ethyl ether, two 5 ml portions of solvent being used. After shaking the solutions with the solvent for 1 minute the tubes were centrifuged at 2,000 r.p.m. for 3 minutes to precipitate the silica gel. The solvent layers were removed by Pasteur pipette and washed with 5 ml of 2 per cent potassium hydrogen carbonate solution before being dried over 3 g of anhydrous sodium sulphate for 1 hour. The solutions were then filtered and the di-ethyl ether removed by rotary evaporation. The esters were dissolved in 1 ml of ice cold hexane for gas chromatographic analysis. It is essential that the hexane is free from aromatic hydrocarbons. The tubes were kept ice cold to prevent hexane evaporation and the sample was analysed immediately.

6. Gas-liquid chromatography

The esters were separated by gas-liquid chromatography on a 1.52 m x 0.64 cm glass column with 20 per cent diethylene glycol succinate as the stationary phase. The column temperature was 190°C and the detector oven temperature 200°C. Nitrogen was

used as the carrier gas at a flow rate of 35 ml/min. The peak areas were measured as peak height x retention time. Weights of lipids were calculated by multiplying the weight of fatty-acid by the appropriate conversion factors according to Christie, et al., (1970).

RESULTS

1. A COMPARISON OF THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE AND CHILL-RESISTANT LEAVES GROWN AT 25°C

The degree of unsaturation of the phospho- and glycolipids was compared by calculating:-

a) The total percentage of unsaturated fatty-acid associated with each lipid.

b) The total percentage of linoleic (18:2) and linolenic (18:3) acid associated with each lipid. This method was adopted as these fatty-acids are the predominant unsaturated fatty-acids in leaves and Lyons and Asmundson (1965) have shown that linoleic acid is as effective as linolenic acid in lowering the temperature of the phase change of mixtures of pure fatty-acids. In addition, they showed that oleic acid (18:1) was not as effective as linoleic and linolenic acid in lowering the transition temperature.

Glycolipids

Tables 20 to 22 show that the total degree of unsaturation and total percentage of linoleic and linolenic acid of the individual glycolipids of Hordeum vulgare is not significantly greater than the chill-sensitive species. However, the lipids MGDG and DGDG from the very chill-sensitive Episcia reptans leaves have approximately

Table 20. A comparison of the percentage fatty-acid composition of monogalactosyl diglyceride in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid*	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	1.4	1.6	1.2	1.4
16:0	1.8	1.8	1.8	1.3
16:1	0.6	0.5	0.4	0.4
16:2	0.6	0.7	0.2	0.4
18:0	0.5	0.5	0.4	0.3
18:1	1.5	0.8	0.4	0.4
18:2	12.4	2.2	1.7	4.1
18:3	81.2	91.9	93.9	91.7
Total per cent 18:2 + 18:3	93.6	94.1	95.6	95.8
Total per cent unsaturated fatty-acid	96.3	96.1	96.6	96.9

*Ratio shown denotes the number of carbon atoms to the number of double bonds in the molecule.

Table 21. A comparison of the percentage fatty-acid composition of digalactosyl diglyceride in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	2.1	2.0	1.2	1.0
16:0	7.7	9.8	9.1	11.1
16:1	1.5	0.6	0.6	0.4
16:2	1.2	0.6	0.4	0.2
18:0	1.4	1.0	2.0	1.0
18:1	2.3	1.6	0.7	0.6
18:2	13.6	2.9	1.6	4.0
18:3	70.2	81.5	84.4	81.7
Total per cent 18:2 + 18:3	83.8	84.4	86.0	85.7
Total per cent unsaturated fatty-acid	88.5	87.2	87.7	86.9

Table 22. A comparison of the percentage fatty-acid composition of sulpholipid in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	12.7	8.9	4.9	7.0
16:0	21.0	33.6	21.7	24.1
16:1	5.7	2.9	2.3	2.1
16:2	4.7	3.9	3.2	1.9
18:0	4.3	3.3	4.8	2.2
18:1	5.2	5.8	2.2	1.8
18:2	13.1	7.1	3.0	5.2
18:3	33.3	34.5	57.9	55.7
Total per cent 18:2 + 18:3	46.4	41.6	60.9	60.9
Total per cent unsaturated fatty-acid	62.0	54.2	68.4	66.7

10 per cent less linolenic acid and 10 per cent more linoleic acid than those from Gossypium hirsutum and Phaseolus vulgaris leaves. Since linoleic acid is considered to have the same effect as linolenic acid in lowering the temperature of the phase change (Lyons and Asmundson, 1965), the lower percentage of linolenic acid associated with the lipids MGDG and DGDG of Episcia reptans leaves is not thought to be related to their greater chill-sensitivity.

Phospholipids

Tables 23 to 27 show that, in general, the total degree of unsaturation (calculation a) of the individual phospholipids of chill-resistant Hordeum vulgare is not significantly greater than chill-sensitive species. Nevertheless, the total percentage of linoleic and linolenic acid (calculation b) associated with each phospholipid is generally higher in the leaves of Hordeum vulgare, especially PE and PG (Tables 24 and 27). In addition, the total percentage of linoleic and linolenic acid associated with PC, PI and PA of Episcia reptans (a species injured at 12°C) was lower than the less chill-sensitive species Gossypium hirsutum and Phaseolus vulgaris (species which are not injured until below 10°C) Tables 23, 25 and 26. However, the total percentage of linoleic and linolenic acid associated with the lipids PE and PG is not precisely correlated with chill-sensitivity in all species as the level of these fatty-acids associated with PE and PG of Episcia reptans is higher than in Gossypium hirsutum (Tables 24 and 27).

Because the percentage fatty-acid composition of the phospholipids is compared a low level of linoleic and linolenic acid must be associated with a high level of other fatty-acids. For example, the percentage of

Table 23. A comparison of the percentage fatty-acid composition of phosphatidyl choline in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	2.6	1.4	1.2	1.4
16:0	15.6	19.7	16.4	24.6
16:1	2.8	1.0	0.6	0.7
16:2	2.9	0.6	0.6	1.1
18:0	7.9	1.6	5.5	1.8
18:1	17.7	18.4	5.4	2.5
18:2	38.3	43.4	34.3	36.8
18:3	12.2	13.9	36.0	31.1
Total per cent 18:2 + 18:3	50.5	57.3	70.3	67.9
Total per cent unsaturated fatty-acid	73.9	77.3	76.9	72.2

Table 24. A comparison of the percentage fatty-acid composition of phosphatidyl ethanolamine in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	6.7	5.7	3.8	5.1
16:0	15.0	26.3	27.2	20.0
16:1	2.8	1.9	2.0	1.9
16:2	3.3	1.7	1.2	3.2
18:0	5.5	2.0	3.7	2.2
18:1	8.8	6.6	2.6	1.7
18:2	45.1	43.5	28.0	41.8
18:3	12.8	12.3	31.5	24.1
Total per cent 18:2 + 18:3	57.9	55.8	59.5	65.9
Total per cent unsaturated fatty-acid	72.8	66.0	65.3	72.7

Table 25. A comparison of the percentage fatty-acid composition of phosphatidyl inositol in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	8.6	6.5	4.3	5.2
16:0	26.4	31.4	31.3	31.3
16:1	6.7	2.7	1.7	3.6
16:2	6.9	3.1	2.4	4.6
18:0	6.7	3.6	5.4	5.4
18:1	8.9	6.0	2.5	3.0
18:2	21.5	32.5	15.7	21.6
18:3	14.3	14.2	36.7	25.3
Total per cent 18:2 + 18:3	35.8	46.7	52.4	46.9
Total per cent unsaturated fatty-acid	58.3	58.4	59.0	58.1

Table 26. A comparison of the percentage fatty-acid composition of phosphatidic acid in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	21.6	10.5	10.1	16.4
16:0	12.0	14.6	13.0	12.9
16:1	6.7	7.3	2.5	5.4
16:2	8.1	-	2.7	10.0
18:0	5.7	6.6	4.8	4.9
18:1	8.6	15.0	6.4	4.2
18:2	21.0	32.0	29.0	23.3
18:3	16.3	14.0	31.5	22.9
Total per cent 18:2 + 18:3	37.3	46.0	60.5	46.2
Total per cent unsaturated fatty-acid	60.7	68.3	72.1	65.8

Table 27. A comparison of the percentage fatty-acid composition of phosphatidyl glycerol in the leaves of chill-sensitive and chill-resistant plants at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	5.8	5.5	3.7	3.2
16:0	22.6	41.7	34.4	8.5
16:1	11.2	10.4	12.9	18.3
16:2	4.2	4.9	4.5	5.2
18:0	4.8	2.9	5.6	1.7
18:1	20.5	8.4	4.8	4.3
18:2	23.2	14.5	9.1	7.8
18:3	7.8	11.7	25.0	51.0
Total per cent 18:2 + 18:3	31.0	26.2	34.1	58.8
Total per cent unsaturated fatty-acid	66.9	49.9	56.3	86.6

Table 28. A comparison of the weights of phospholipid in the leaves of chill-sensitive and chill-resistant plants grown at 25°C (each lipid expressed as a percentage of the total weight of phospholipid)

Phospholipid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
PC	52.2	53.7	42.9	42.6
PE	25.0	21.4	21.2	19.3
PI	7.1	5.4	6.8	4.5
PA	4.7	5.7	5.0	3.1
PG	10.9	13.8	24.1	30.5

linoleic and linolenic acid associated with the phospholipids of Episcia reptans is low and a high level of oleic acid (18:1) is present (Tables 23 to 27). The close relationship between chill-sensitivity and the total percentage of linoleic and linolenic acid of the phospholipids and not the total degree of unsaturation is attributable mainly to a low level of linoleic and linolenic acid and hence a high level of oleic acid associated with the phospholipids of Episcia reptans and Gossypium hirsutum. Furthermore, the level of oleic acid is particularly high in PC of these species (Table 23) and PC comprises 52 per cent of the total weight of phospholipid in comparison to 42 per cent in Hordeum vulgare and Phaseolus vulgaris (Table 28). Although Phaseolus vulgaris is chill-sensitive, it can be regarded as a 'borderline' species in terms of the degree of unsaturation of the phospholipids since the total percentage of linoleic and linolenic acid associated with the lipids PC, PI and PA is higher than Hordeum vulgare (Tables 23, 25 and 26).

The total percentage of linoleic and linolenic acid associated with the phospholipids was generally highest in chill-resistant Hordeum vulgare and lowest in the very chill-sensitive species Episcia reptans so that a comparison of the total percentage of linoleic and linolenic acid of all the phospholipids combined showed the best relationship to chill-sensitivity (Table 29). In contrast, no relationship was detected when the total percentage of linoleic and linolenic acid of all the glycolipids combined was compared (Table 30). Due to differences in the weight of glycolipid present in the leaves of each species (Table 31) the relationship between the total percentage of linoleic and linolenic acid of all the polar lipids (i.e. phospho- and glycolipids combined) and

Table 29. A comparison of the total percentage fatty-acid composition of all the phospholipids in the leaves of chill-sensitive and chill-resistant plants grown at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	5.0	3.1	2.9	3.8
16:0	16.4	25.1	22.9	19.1
16:1	3.8	2.7	3.9	7.7
16:2	3.5	2.1	1.8	2.3
18:0	6.9	2.2	4.9	1.8
18:1	14.8	12.8	4.3	2.4
18:2	37.4	35.8	28.6	24.7
18:3	12.2	16.2	30.7	38.2
Total per cent 18:2 + 18:3	49.6	52.0	59.3	62.9
Total per cent unsaturated fatty-acid	71.7	69.6	69.3	75.3

Table 30. A comparison of the total percentage fatty-acid composition of all glycolipids in the leaves of chill-sensitive and chill-resistant plants grown at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	2.0	1.5	1.3	1.8
16:0	4.6	5.4	5.1	8.0
16:1	1.9	0.6	0.6	0.6
16:2	0.9	0.8	0.6	0.5
18:0	1.0	0.7	1.1	0.9
18:1	2.0	1.5	0.9	0.8
18:2	13.0	3.2	2.2	6.5
18:3	75.6	86.3	88.2	80.9
Total per cent 18:2 + 18:3	88.6	89.5	90.4	87.4
Total per cent unsaturated fatty-acid	93.4	92.4	92.5	89.3

Table 31. A comparison of the weights of polar lipids in the leaves of chill-sensitive and resistant plants at 25°C (i.e. each lipid expressed as a percentage of the total weight of lipid)

Lipid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
MGDG	40.1	30.3	33.0	45.
DGDG	25.3	18.0	21.5	22.4
SL	1.9	2.8	2.0	2.0
PC	17.1	26.2	18.7	12.5
PE	8.2	10.3	9.2	5.4
PI	2.3	3.5	3.0	1.7
PG	3.6	6.5	10.4	9.5
PA	1.5	2.4	2.2	1.5
Total per cent phospholipid	32.7	48.9	43.5	30.6

Table 32. A comparison of the total percentage fatty-acid composition of the phospho- and glycolipids (i.e. all the polar lipids) in the leaves of chill-sensitive and resistant plants grown at 25°C

Fatty-acid	Chill-sensitive			Chill-resistant
	Episcia reptans	Gossypium hirsutum	Phaseolus vulgaris	Hordeum vulgare
14:0	2.9	2.6	2.2	1.8
16:0	7.9	13.7	12.4	8.8
16:1	1.9	1.5	1.8	2.1
16:2	1.7	1.5	1.1	0.8
18:0	2.5	1.4	2.6	0.9
18:1	5.4	5.8	2.2	1.0
18:2	19.3	16.5	10.9	9.5
18:3	58.4	57.0	66.8	75.1
Total per cent 18:2 + 18:3	77.7	73.5	77.7	84.6
Total per cent unsaturated fatty-acid	86.7	82.3	82.8	88.5

chill-sensitivity is not as precise as comparing the total percentage of linoleic and linolenic acid of all the phospholipids combined (Tables 32 and 29 respectively). For example, because Episcia reptans has high levels of glycolipid (Table 31) the total percentage of linoleic and linolenic acid of all the polar lipids combined is higher than in Gossypium hirsutum (Table 32).

Lyons et al (1964) analysed the total fatty-acid composition of mitochondria isolated from a number of chill-sensitive and chill-resistant fruits and their results are presented in Table 33. They showed that a significant relationship exists between the total percentage of unsaturated fatty-acid and chill-sensitivity. In the present thesis their results are used to compare the total percentage of linoleic and linolenic acid associated with the mitochondria of fruits (Table 33) with the results obtained above for leaves. In contrast to leaves, the chill-sensitivity of mitochondria isolated from fruits is associated best with the total percentage of unsaturated fatty-acid and not the total percentage of linoleic and linolenic acid. Table 33 shows that the relationship between a high total percentage of unsaturated fatty-acid and chill-resistance of fruits is dependent mainly on a high level of oleic acid associated with the mitochondria of chill-resistant cauliflower buds and turnip roots. Therefore a high level of oleic acid is generally associated with mitochondria from chill-resistant fruits and with the phospholipids from chill-sensitive leaves (P. 119). This discrepancy can only be resolved by analysing the fatty-acid compositions of individual membrane lipid components of mitochondria isolated from fruits. It is interesting to note that the total degree of unsaturation of the mitochondrial fatty-acid from

Phaseolus vulgaris shoots is 'borderline' between chill-sensitivity and chill-resistance - a similar relationship to that which was found to exist in leaves (P. 119).

Chill-sensitivity could not be related to the lipid composition of the leaves. Table 31 shows that Hordeum vulgare leaves contain a lower weight of phospholipid than chill-sensitive species.

In conclusion, the chill-sensitivity of leaves was best related to the total percentage of linoleic and linolenic acid associated with each phospholipid and not the total degree of unsaturation of each phospholipid. Therefore a comparison of the total percentage of linoleic and linolenic acid of all the phospholipids combined showed the most precise relationship to the chill-sensitivity of the species (Fig. 11). In contrast, the chill-sensitivity of mitochondria isolated from fruits appears to be related best to the total degree of unsaturation of the mitochondrial fatty-acids and not the total percentage of linoleic and linolenic acid.

Table 33. The fatty-acid composition of plant mitochondria isolated from fruits, storage organs and shoots

Data from Lyons et al. (1964).

Fatty-acid	Relative retention	Chill-resistant			Chill-sensitive			
		Cauliflower bud	Turnip root	Pea shoot	Bean shoot	Sweet potato root	Corn shoot	Green tomato shoot
12:0	0.33	-	-	-	-	8.4	-	-
16:0	1.00	21.3	19.0	17.8	24.0	24.9	28.3	22.5
16:1	1.13	0.8	1.3	0.4	0.4	0.3	0.8	0.6
17:0	1.28	-	-	-	0.4	-	-	-
	1.48	-	0.4	-	0.2	-	0.4	0.6
18:0	1.68	1.9	1.1	2.9	2.2	2.6	1.6	2.5
	1.74	0.8	-	-	-	-	-	-
18:1	1.95	7.0	12.2	3.1	3.8	0.6	4.6	2.2
18:2	2.42	16.1	20.6	61.9	43.6	50.8	54.6	44.9
18:3	3.07	49.4	44.9	13.2	24.3	10.6	6.8	21.5
	3.20	-	-	-	-	-	0.7	-
22:0	4.98	-	-	-	-	-	1.5	-
Total per cent 18:2 + 18:3		65.5	65.5	75.1	67.9	61.4	61.4	66.4
Total per cent unsaturated fatty-acid		73.3	79.0	78.6	72.1	62.3	66.8	69.2

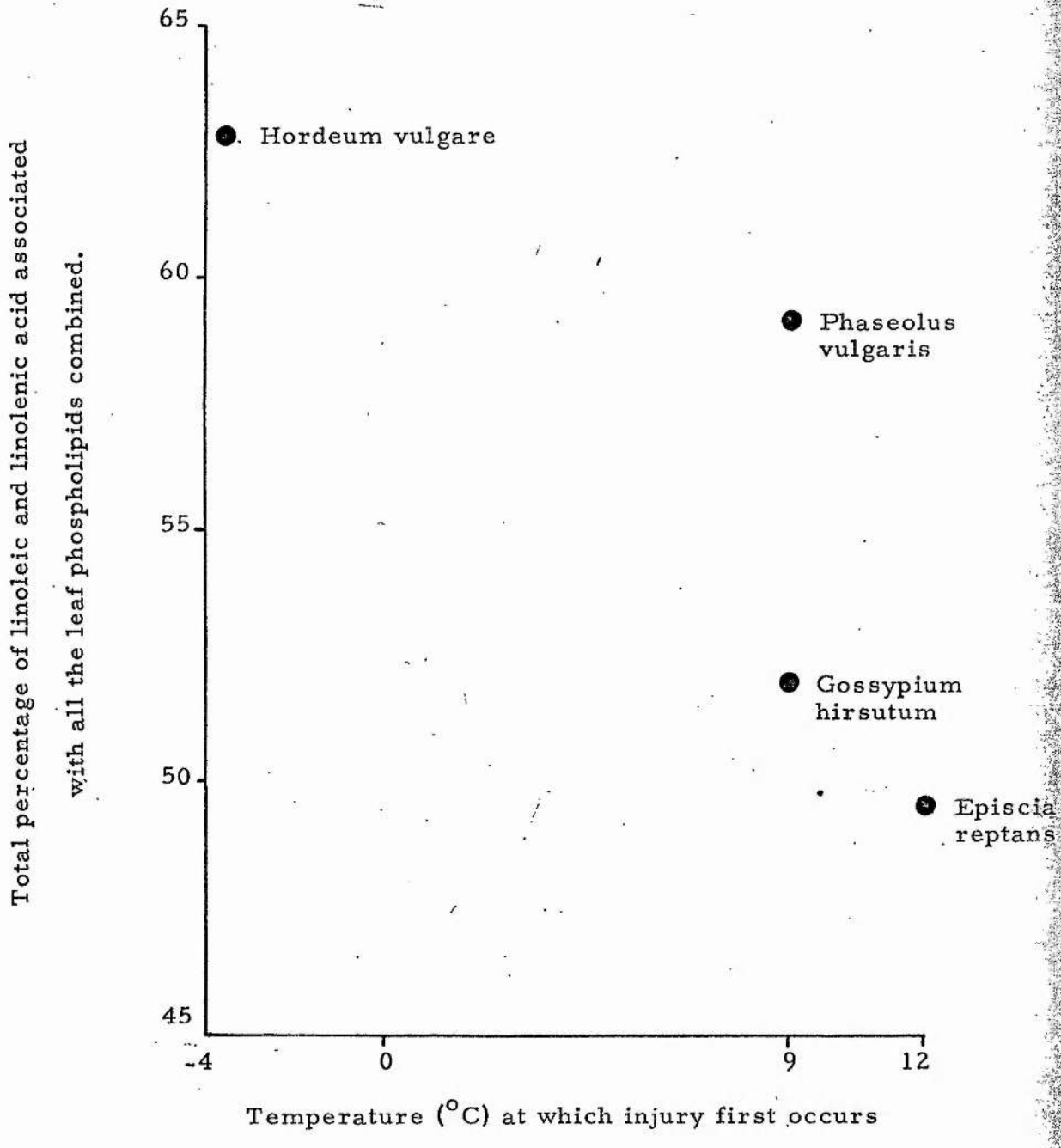


Fig. 11. A comparison of the total percentage of linoleic and linolenic acid associated with all the leaf phospholipids combined and the temperature at which chilling injury first occurs. Leaves were grown at 25°C, 95 per cent RH.

2. THE EFFECTS OF HARDENING

A) The effect of hardening and leaf age on the susceptibility of chill-resistant Hordeum vulgare to freezing injury at -4°C

Hardening Hordeum vulgare for 1 day at 5°C, 85 per cent RH, or 4 days at 12°C, 95 per cent RH, against freezing-injury at -4°C was not as effective as the hardening of chill-sensitive species against chilling-injury. The most effective hardening treatment of Hordeum vulgare used in this investigation was 1 day at 5°C but 48 per cent of these plants died after 24 hours of frost. Hardening for 4 days at 12°C was less effective and 67 per cent of the plants died after 24 hours frost (Table 34). Nevertheless, hardening Hordeum vulgare for 1 day at 5°C or 4 days at 12°C provided a significant degree of protection against freezing-injury in comparison to plants transferred directly from 25 to -4°C (Table 34). Similarly, Levitt (1969) reported that the exposure of cabbage leaves to 5°C for only 1 day provided a marked degree of protection against freezing-injury.

In Chapter 1 it was reported that older leaves of chill-sensitive Gossypium hirsutum and Phaseolus vulgaris were more susceptible to chilling-injury than younger leaves. Similarly, in this study of freezing-injury in Hordeum vulgare, it was discovered that 8-day-old leaves grown at 25°C were 2 to 3 times more susceptible to freezing-injury than 4-day-old leaves (Table 34).

Table 34. The effect of leaf age and hardening for 4 days at 12°C, 95 per cent RH, or 1 day at 5°C on the susceptibility of Hordeum vulgare to freezing-injury at -4°C in the dark. The number of plants dead due to freezing was recorded after 3 days recovery at 15 to 20°C. Each value is based on 100 plants.

Hours at -4°C	Percentage of plants dead			
	Non-hardened leaves		Hardened leaves (leaves 4 days old at the start of hardening)	
	Leaves 4 days old	Leaves 8 days old	Hardened for 4 days at 12°C, 95 per cent RH, before freezing	Hardened for 1 day at 5°C, 85 per cent RH, before freezing
8	3	9	2	0
13	30	62	25	8
24	97	100	67	48

B) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive and chill-resistant species on hardening at 12°C, 95 per cent RH.

Chill-sensitive species

Hardening the 3-day-old leaves of Phaseolus vulgaris and 5-day-old leaves of Gossypium hirsutum for 4 days at 12°C, 95 per cent RH, in the light provided 95 per cent protection against subsequent chilling at 5°C, 85 per cent RH (P. 40). This hardening treatment produced increases of up to 12 per cent in the percentage of linoleic acid (Fig. 12) and hence total per cent unsaturated fatty-acid (Table 35) in all the phospholipids of Gossypium hirsutum and Phaseolus vulgaris leaves. The increase in linoleic acid (18:2) during hardening was accompanied by a decrease in the percentage of palmitic (16:0) and oleic acids (18:1), whilst the percentage of linolenic acid (18:3) remained constant. Most of the increase in linoleic acid occurred after 2 days at the hardening temperature, a period which provided nearly complete protection against chilling-injury (P. 40). The increases in linoleic acid were particularly large in PC, PE and PI which are considered to be important constituents of the lipid portion of the plasmallema and microsomal fractions (Table 19). When total leaf fatty-acid was analysed the increase in linoleic acid detected was small (Chapter 3) due to the fact that only 22 per cent of the total leaf fatty-acids are associated with the phospholipids (Table 3). No increases in either linoleic acid or total per cent unsaturated fatty-acid occurred during the ineffective hardening of Episcia reptans at 15°C (Fig. 12 and Table 35 respectively), except for

a small increase in total per cent unsaturated fatty-acid in PC. This result indicates that increases in unsaturation of the fatty-acids in Gossypium hirsutum and Phaseolus vulgaris during the hardening period are not due to an indirect effect of temperature on lipid metabolism unassociated with the development of chilling-resistance.

During the 4-day period at 12°C the chill-resistant species, Hordeum vulgare, showed no significant change in fatty-acid composition of the phospholipids, except for small increases in total per cent unsaturated fatty-acid in PC, PE and PA (Table 35). However, in the control chill-sensitive and chill-resistant plants maintained at 25°C over the hardening period, the percentage of linoleic acid and total per cent unsaturated fatty-acid decreased (Fig. 12). The decrease in the percentage of linoleic acid over 4 days at 25°C was accompanied by an increase in the percentage of palmitic acid. Therefore in the chill-sensitive plants Gossypium hirsutum and Phaseolus vulgaris, acclimatization results in large increases in unsaturation above the level at the start of hardening, whereas in chill-resistant plants hardening only prevents the decrease in unsaturation that occurs with increase in physiological age at 25°C.

The decreases in unsaturation of the phospholipids of chill-sensitive plants with increase in age at 25°C could be related to the greater susceptibility of older leaves to damage at chilling and hardening temperatures. Table 8 (Chapter 1) compares the degree

of damage due to chilling the leaves of Gossypium hirsutum and Phaseolus vulgaris, which had been grown for 5 and 3 days respectively at 25°C, with the degree of damage on chilling the controls which had been maintained at 25°C for a further 4 days before chilling. The older leaves sustained nearly twice as much damage as the younger ones.

No changes were detected in the fatty-acid composition of the glycolipids MGDG, DGDG and SL in the chill-sensitive plants at 12°C or in the controls maintained at 25°C (Tables 36, 37 and 38).

Chill-resistant species

There was no significant change in the fatty-acid composition of the phospholipids of Hordeum vulgare during hardening at 12°C (Fig. 12), except for small increases in total per cent unsaturated fatty-acid in PC, PE and PA (Table 35). These increases are associated with the increased resistance of the plants to frost-injury at -4°C (Table 34).

The degree of unsaturation of the phospholipids of Hordeum vulgare decreased with increase in age at 25°C (Fig. 12). These decreases could be related to the greater susceptibility of older plants to frost-injury. Table 34 shows that 8-day-old leaves of Hordeum vulgare were 2 to 3 times more injured than 4-day-old leaves.

No changes were detected in the fatty-acid composition of the glycolipids MGDG, DGDG and SL in Hordeum vulgare at 12°C or in the controls maintained at 25°C (Tables 36, 37 and 38).

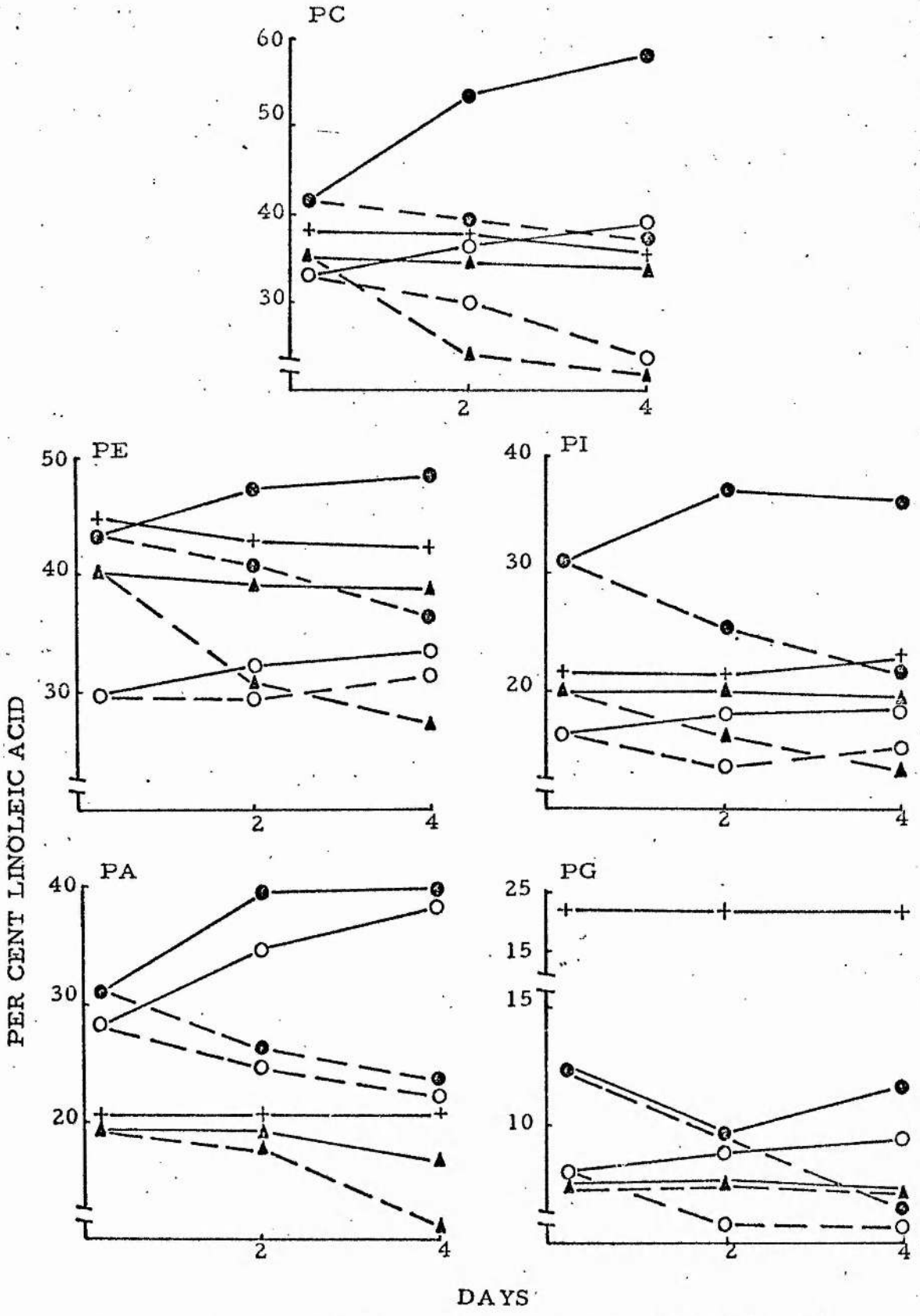


Fig. 12. The effect of hardening on the percentage of linoleic acid associated with phospholipids. Continuous lines indicate plants hardened at 12°C, 95 per cent RH (15°C in the case of Episcia reptans), and broken lines controls which remained at 25°C.
● Gossypium hirsutum; O Phaseolus vulgaris; + Episcia reptans; ▲ Hordeum vulgare.

Table 35. Changes in the total per cent unsaturated fatty-acid associated with the phospholipids on hardening at 12°C (15°C in the case of Episcia reptans). The degree of unsaturation of the controls after 4 days at 25°C is compared with the hardened plants after 4 days at 12°C

Lipid	Total per cent unsaturated fatty-acid							
	Episcia reptans		Gossypium hirsutum		Phaseolus vulgaris		Hordeum vulgare	
	25°C	15°C	25°C	12°C	25°C	12°C	25°C	12°C
PC	73.8	74.8	70.6	81.3	69.3	80.8	71.2	74.1
PE	72.8	71.1	60.9	67.7	63.2	66.8	70.1	73.3
PI	58.3	57.6	52.6	60.6	69.4	64.7	58.4	59.9
PA	60.7	52.0	62.6	72.4	66.9	80.8	62.6	65.4
PG	66.8	61.8	45.2	55.3	54.4	59.7	85.8	85.6

Table 36. A comparison of the percentage fatty-acid composition of monogalactosyl diglyceride in the leaves of chill-sensitive and chill-resistant plants after four days hardening at 12°C, 95 per cent RH (15°C in the case of Episcia reptans)

Fatty-acid	Chill-sensitive						Chill-resistant	
	Episcia reptans		Gossypium hirsutum		Phaseolus vulgaris		Hordeum vulgare	
	Control	Hardened	Control	Hardened	Control	Hardened	Control	Hardened
14:0	1.4	2.4	1.6	1.2	1.2	1.2	1.4	1.0
16:0	1.8	1.8	1.8	1.1	1.8	1.0	1.3	1.4
16:1	0.6	0.6	0.5	0.3	0.4	0.4	0.4	0.2
16:2	0.6	0.5	0.7	0.5	0.2	0.3	0.4	0.3
18:0	0.5	0.6	0.5	0.4	0.4	0.2	0.3	0.2
18:1	1.5	1.6	0.8	0.7	0.4	0.4	0.4	0.3
18:2	12.4	10.5	2.2	2.3	1.7	1.9	4.1	3.2
18:3	81.2	82.0	91.9	93.5	93.9	94.6	91.7	93.4
Total per cent 18:2 + 18:3	93.6	92.5	94.1	95.8	95.6	96.5	95.8	96.6
Total per cent unsaturated fatty-acid	96.3	95.2	96.1	97.4	96.6	97.6	97.0	97.4

Table 37. A comparison of the percentage fatty-acid composition of digalactosyl diglyceride in the leaves of chill-sensitive and chill-resistant plants after four days hardening at 12°C, 95 per cent RH (15°C in the case of Episcia reptans)

Fatty-acid	Chill-sensitive						Chill-resistant	
	Episcia reptans		Gossypium hirsutum		Phaseolus vulgaris		Hordeum vulgare	
	Control	Hardened	Control	Hardened	Control	Hardened	Control	Hardened
14:0	2.1	2.1	2.0	1.4	1.2	0.9	1.0	0.8
16:0	7.7	7.3	9.8	8.5	9.1	7.8	11.1	12.9
16:1	1.5	1.3	0.6	0.5	0.6	0.7	0.4	0.3
16:2	1.2	1.1	0.6	0.7	0.4	0.6	0.2	0.3
18:0	1.4	1.4	1.0	0.8	2.0	1.7	1.0	1.0
18:1	2.3	2.5	1.6	1.4	0.7	0.8	0.6	0.6
18:2	13.6	13.6	2.9	2.8	1.6	1.3	4.0	3.3
18:3	70.2	70.7	81.5	83.9	84.4	86.2	81.7	80.8
Total per cent 18:2 + 18:3	83.8	84.3	84.4	86.7	86.0	87.5	85.7	84.1
Total per cent unsaturated fatty-acid	88.8	89.2	87.2	89.2	87.7	89.6	86.9	85.3

Table 38. A comparison of the percentage fatty-acid composition of sulpholipid in the leaves of chill-sensitive and chill-resistant plants after four days hardening at 12°C, 95 per cent RH (15°C in the case of Episcia reptans)

Fatty-acid	Chill-sensitive						Chill-resistant	
	Episcia reptans		Gossypium hirsutum		Phaseolus vulgaris		Hordeum vulgare	
	Control	Hardened	Control	Hardened	Control	Hardened	Control	Hardened
14:0	12.7	9.4	8.9	5.6	4.9	5.3	7.0	6.4
16:0	21.0	21.2	33.6	34.3	21.7	22.3	24.1	22.4
16:1	5.7	6.4	2.9	1.3	2.3	2.6	2.1	2.0
16:2	4.7	3.5	3.9	2.9	3.2	2.5	1.9	2.7
18:0	4.3	3.0	3.3	2.3	4.8	4.7	2.2	2.1
18:1	5.2	4.4	5.8	4.0	2.2	1.8	1.8	1.8
18:2	13.1	13.7	7.1	4.9	3.0	2.1	5.2	4.6
18:3	33.3	38.4	34.5	44.7	57.9	58.7	55.7	58.0
Total per cent 18:2 + 18:3	46.4	52.1	41.6	49.6	60.9	60.8	60.9	62.6
Total per cent unsaturated fatty-acid	62.0	66.4	54.2	57.8	68.4	67.7	66.6	69.1

C) Changes in the weights of the polar lipids of chill-sensitive and chill-resistant species on hardening at 12°C, 95 per cent RH

Chill-sensitive species

The weights of all phospholipids remained higher during the hardening of Gossypium hirsutum and Phaseolus vulgaris than in the controls maintained at 25°C (Fig. 13). However, the weights of all phospholipids decreased with the development of the plants at 12 and 25°C (except for increases in the weights of PI in Gossypium hirsutum and PA in Phaseolus vulgaris), the rate of decrease being slower in the hardening plants. This result is contrary to that of Guinn (1971) who reported a decrease in lipid soluble phosphorous on hardening Gossypium hirsutum. The different results of Guinn may be explained by the wide fluctuation in lipid soluble phosphorous content of his control plants.

The low levels of phospholipid in the older leaves of chill-sensitive plants at 25°C, combined with the lower degree of unsaturation of the fatty-acids, may play an important role in determining the degree of injury suffered by a leaf at a particular age. The absolute decrease in the weight of phospholipids per gramme dry weight of leaf in the control chill-sensitive species may, however, be due to the storage of carbohydrates synthesized under the favourable growth conditions.

The weights of all glycolipids also tended to decrease in the chill-sensitive plants at 12 and 25°C, except for a small increase

in MGDG in Phaseolus vulgaris at 25°C (Fig. 14). The weights of the glycolipids MGDG and DGDG were lower in the hardened chill-sensitive plants than in the controls, except for DGDG in Gossypium hirsutum. If hardening involves resynthesis of phospholipids with a higher degree of unsaturation, it is thought that these decreases in glycolipids might be due to their catabolism either as a source of energy or of fatty-acids. Since the weight of phospholipids is higher in the hardened chill-sensitive plants, the decrease in total weight of leaf fatty-acid on hardening (P. 80) must be attributable to decreases in the weights of neutral and glycolipids.

Chill-resistant species

In chill-resistant Hordeum vulgare the weights of the phospholipids PC, PE and PG increased at 12°C and the weights of all the phospholipids decreased in the controls maintained at 25°C (Fig. 13). The increase in weight of these phospholipids and the increase in total per cent unsaturated fatty-acid of PC, PE and PA at 12°C is related to greater resistance of these plants to frost-injury (Table 34).

In contrast to the chill-sensitive species the weights of MGDG in Hordeum vulgare remained very stable at 12 and 25°C and DGDG increased slightly (Fig. 14).

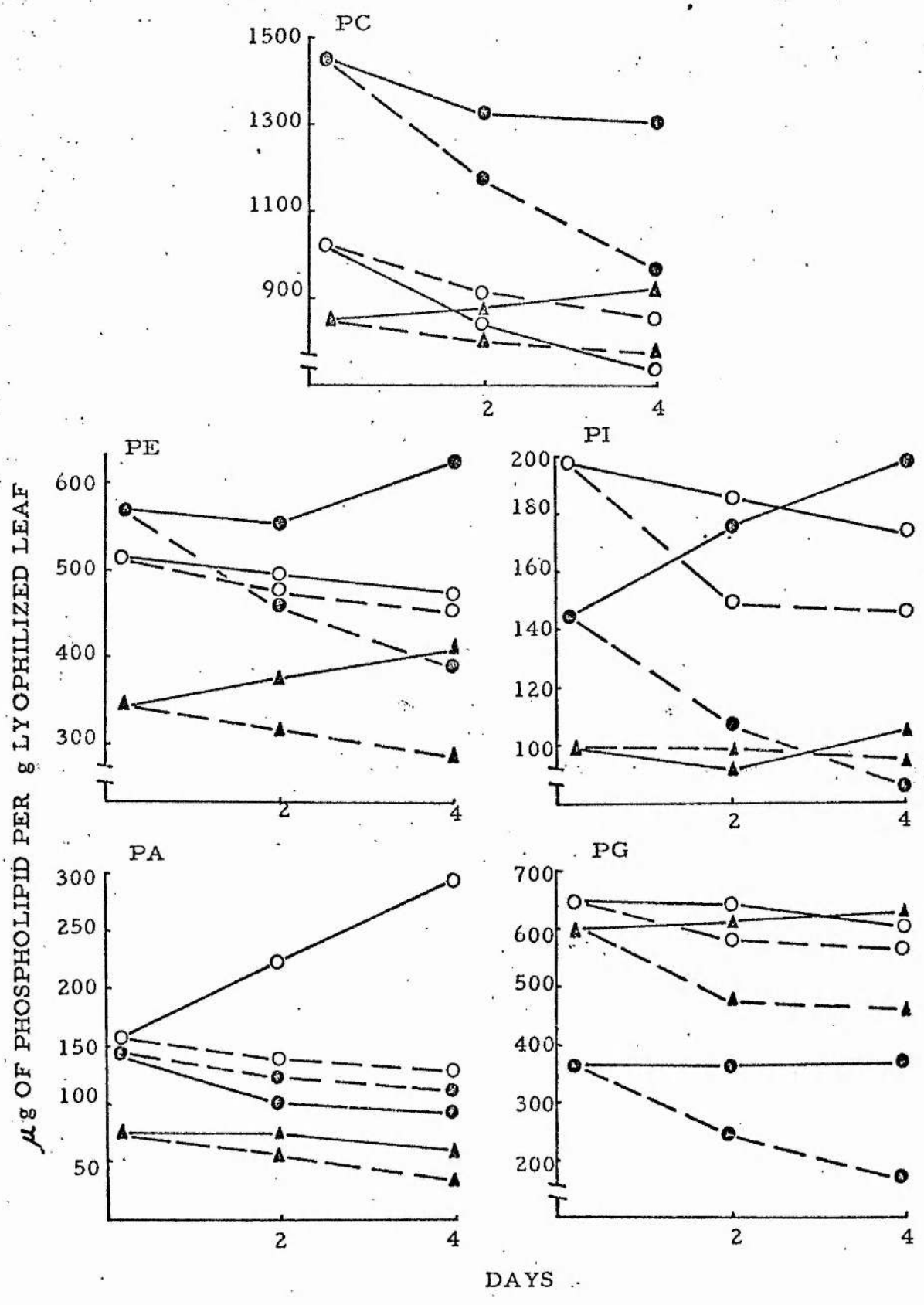


Fig. 13. The effect of hardening on the weight of leaf phospholipids. Continuous lines indicate plants hardened at 12°C, 95 per cent RH, and broken lines controls which remained at 25°C. ● Gossypium hirsutum; ○ Phaseolus vulgaris; ▲ Hordeum vulgare.

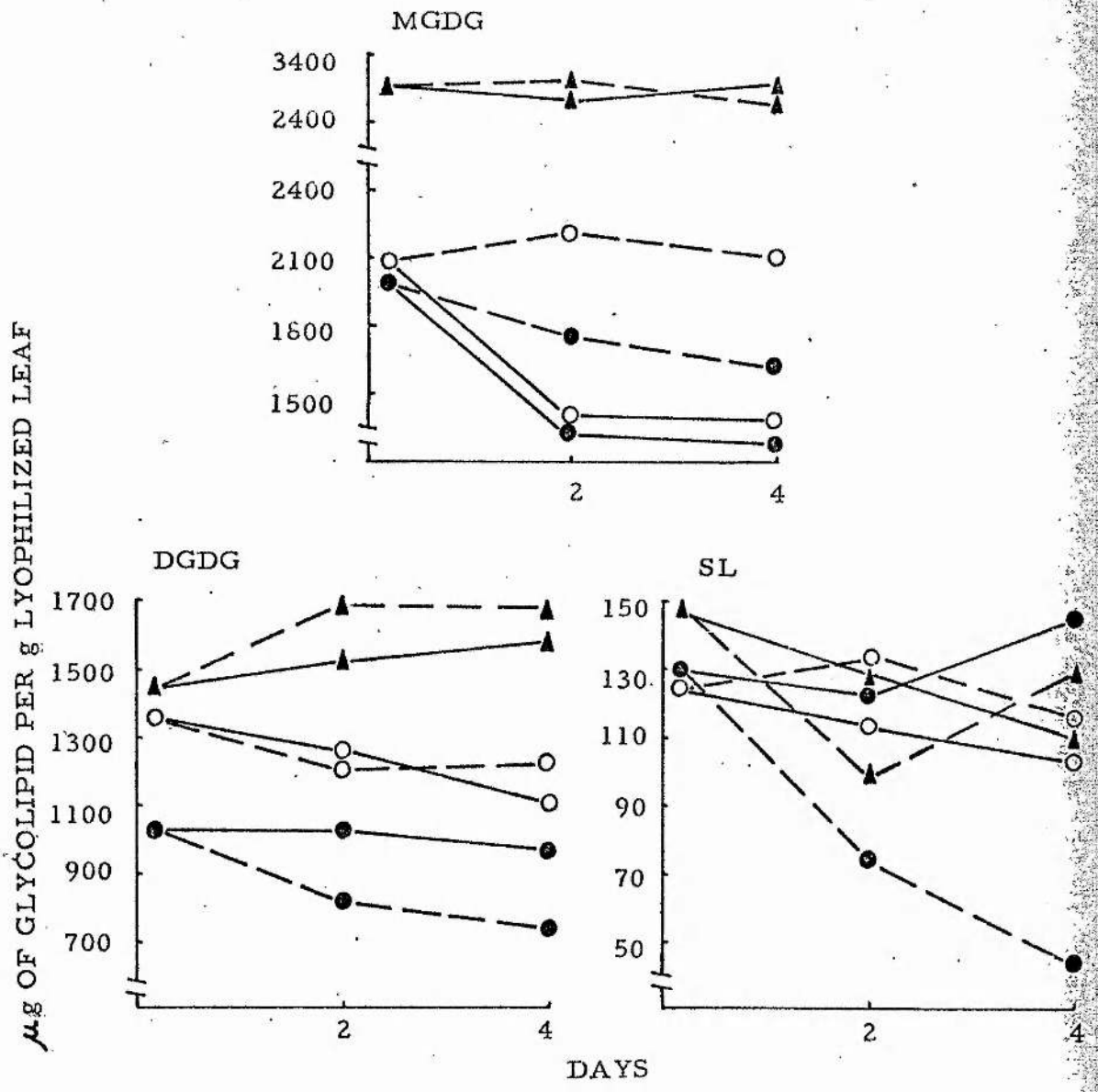


Fig. 14. The effect of hardening on the weight of leaf glycolipids. Continuous lines indicate plants hardened at 12°C, 95 per cent RH, and broken lines controls which remained at 25°C. ● Gossypium hirsutum; ○ Phaseolus vulgaris; ▲ Hordeum vulgare.

3. THE EFFECTS OF CHILLING AT 5°C, 85 PER CENT RH

A) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive and chill-resistant species on chilling at 5°C, 85 per cent RH

Chill-sensitive species

The chilling of hardened and non-hardened Gossypium hirsutum and Phaseolus vulgaris plants resulted in a decrease in the percentage of linoleic acid associated with the phospholipids (Fig. 15), except in PG. These changes are small in comparison to the large increases in unsaturation that occurred on hardening, so that hardened and chilled chill-sensitive plants still have a far greater degree of unsaturation of the phospholipids than the non-hardened and chilled plants.

There was no decrease in the percentage of linolenic acid associated with any of the phospholipids of Gossypium hirsutum and Phaseolus vulgaris on chilling as would be expected from the decrease in the percentage of linolenic acid in the total leaf fatty-acid analyses during chilling (Chapter 3). A decrease in the percentage of linolenic acid associated with the glycolipids MGDG, DGDG and SL occurred on chilling non-hardened Gossypium hirsutum (Table 39) but was not detected in Phaseolus vulgaris (Table 40) and Hordeum vulgare (Table 41). This decrease in the percentage of linolenic acid associated with the glycolipids on chilling Gossypium hirsutum was reduced by hardening the plants before chilling (Table 39). The decrease in the percentage of linolenic acid on chilling non-hardened Gossypium hirsutum was also evident in the calculation of the total fatty-acid composition of the polar lipids and was due, in part, to more pronounced

decreases in the weights of MGDG and PC than other lipids on chilling (Table 42), as well as a decrease in the percentage of linolenic acid associated with the glycolipids (Table 39). These changes did not occur in Phaseolus vulgaris (Table 43).

Chill-resistant species

Hardening Hordeum vulgare for 1 day at 5°C resulted in small increases in the percentage of linoleic acid and hence total per cent unsaturated fatty-acid in the phospholipids PC, PE and PI (Fig. 15). The increase in the percentage of linoleic acid was not detectable during hardening at 12°C which may be the cause of the greater susceptibility of these plants to freezing-injury than those hardened for 1 day at 5°C (Table 34).

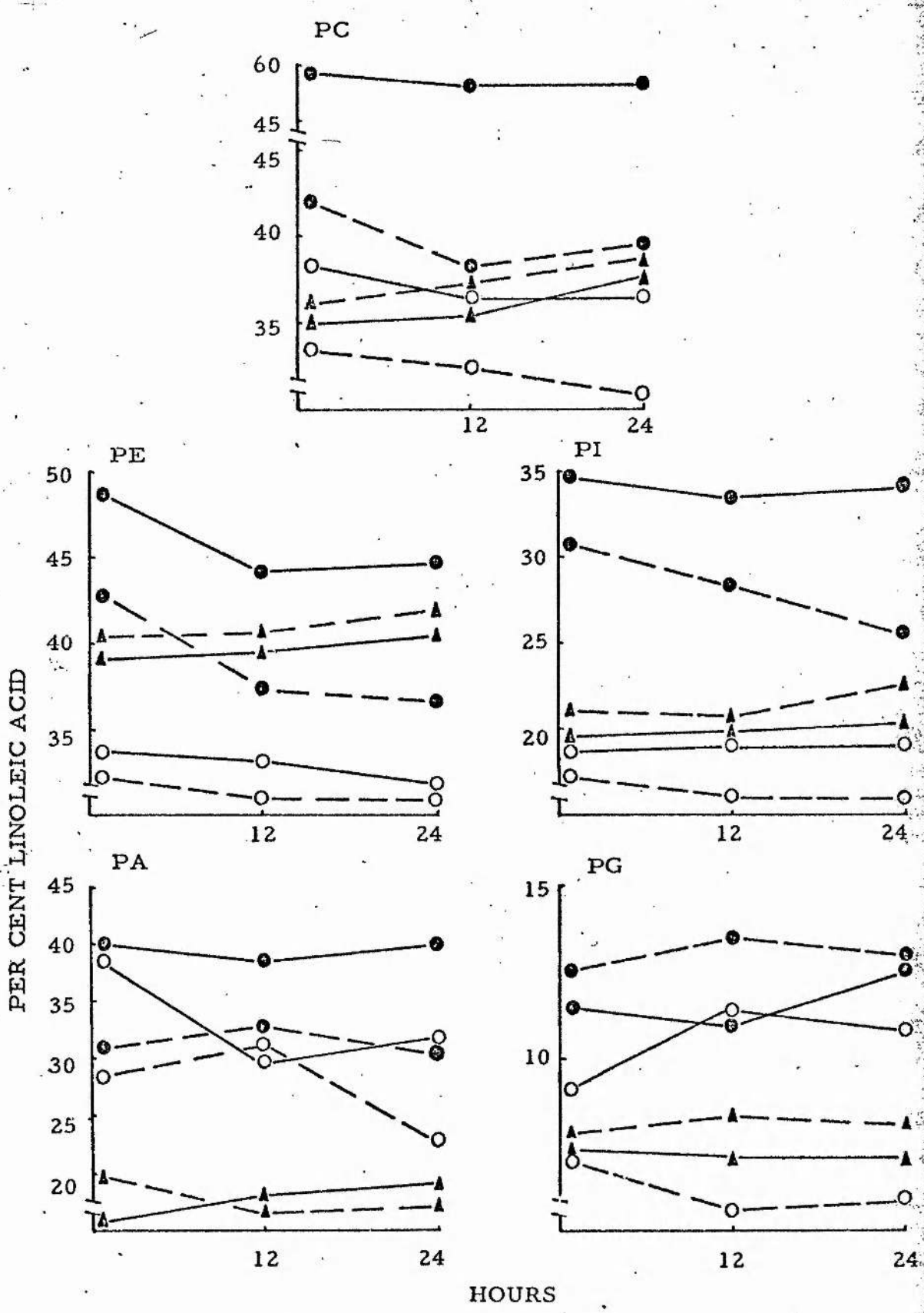


Fig. 15. The effect of chilling hardened and non-hardened plants at 5°C, 85 per cent RH, on the percentage of linoleic acid associated with the leaf phospholipids. Continuous lines indicate plants which had been hardened for 4 days at 12°C, 95 per cent RH, before chilling, and broken lines controls which remained at 25°C before chilling.
● Gossypium hirsutum; O Phaseolus vulgaris; ▲ Hordeum vulgare.

Table 39. Changes in the percentage fatty-acid composition of monogalactosyl diglyceride and digalactosyl diglyceride in the non-hardened and hardened leaves of Gossypium hirsutum during chilling at 5°C, 85 per cent RH.

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
MGDG					
14:0	1.6	1.1	3.2	2.0	2.2
16:0	1.8	1.8	3.5	1.7	2.1
16:1	0.5	0.5	1.1	1.0	0.7
16:2	0.7	0.6	1.7	1.0	1.1
18:0	0.5	0.4	1.1	0.7	0.7
18:1	0.8	1.2	1.7	0.9	0.9
18:2	2.2	2.3	3.2	2.2	2.4
18:3	91.9	92.1	84.5	90.4	89.9
Total per cent unsaturated fatty-acid	96.1	96.7	92.2	95.6	95.0
DGDG					
14:0	2.0	1.7	2.4	2.2	1.6
16:0	9.8	9.0	11.7	10.6	11.2
16:1	0.6	0.7	0.9	0.9	0.6
16:2	0.6	0.7	1.3	1.0	1.0
18:0	1.0	1.1	1.4	1.2	1.0
18:1	1.6	1.7	2.0	1.7	1.6
18:2	2.9	2.7	3.1	3.4	2.6
18:3	81.5	82.4	77.2	79.0	80.4
Total per cent unsaturated fatty-acid	87.2	88.2	84.5	86.0	86.2

Table 39. (Continued). Changes in the percentage fatty-acid composition of sulpholipid in the non-hardened and hardened leaves of Gossypium hirsutum during chilling at 5°C, 85 per cent RH

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
SL					
14:0	8.9	7.6	13.7	6.8	7.7
16:0	33.6	30.1	35.2	31.4	35.0
16:1	2.9	5.7	3.9	4.7	2.8
16:2	3.9	6.3	6.5	5.3	4.9
18:0	3.3	5.3	6.5	6.0	5.3
18:1	5.8	7.1	5.6	6.1	5.3
18:2	7.1	6.6	5.2	6.7	6.0
18:3	34.5	31.3	23.4	33.0	33.0
Total per cent unsaturated fatty-acid	54.2	56.9	44.6	55.8	52.0

Table 40. Changes in the percentage fatty-acid composition of monogalactosyl diglyceride and digalactosyl diglyceride in the non-hardened and hardened leaves of Phaseolus vulgaris during chilling at 5°C, 85 per cent RH

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
MGDG					
14:0	1.2	1.2	1.6	1.7	1.5
16:0	1.8	1.6	2.0	1.1	1.1
16:1	0.4	0.5	0.5	0.4	0.6
16:2	0.2	0.3	0.4	0.2	0.5
18:0	0.4	0.4	0.5	0.3	0.4
18:1	0.4	0.5	0.6	0.5	0.5
18:2	1.7	1.6	1.7	1.9	1.4
18:3	93.9	93.9	92.7	93.9	94.0
Total per cent unsaturated fatty-acid	96.6	96.8	95.9	96.9	97.0
DGDG					
14:0	1.2	1.1	1.0	1.1	0.8
16:0	9.1	9.3	8.8	8.1	8.0
16:1	0.6	0.7	0.4	0.6	0.8
16:2	0.4	0.4	0.3	0.3	0.5
18:0	2.2	1.8	1.8	1.5	1.5
18:1	0.7	0.8	0.5	0.6	0.7
18:2	1.6	1.4	1.1	1.2	1.4
18:3	84.4	84.5	86.1	86.6	86.3
Total per cent unsaturated fatty-acid	87.7	87.8	88.4	89.3	89.7

Table 40. (Continued). Changes in the percentage fatty-acid composition of sulpholipid in the non-hardened and hardened leaves of Phaseolus vulgaris during chilling at 5°C, 85 per cent RH

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
SL					
14:0	4.9	3.8	4.3	3.0	6.0
16:0	21.9	22.8	23.4	23.2	22.1
16:1	2.3	2.2	2.6	2.7	3.1
16:2	3.2	1.8	2.3	2.1	2.6
18:0	4.8	5.3	4.7	4.2	5.4
18:1	2.2	2.2	2.5	1.0	2.0
18:2	3.0	2.6	2.8	1.5	1.8
18:3	57.9	59.3	57.4	62.3	57.0
Total per cent unsaturated fatty-acid	68.4	68.1	67.6	69.6	66.5

Table 41. Changes in the percentage fatty-acid composition of monogalactosyl diglyceride and digalactosyl diglyceride in the non-hardened and hardened leaves of Hordeum vulgare during chilling at 5°C, 85 per cent RH

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
MGDG					
14:0	1.4	1.1	1.1	1.1	1.2
16:0	1.3	1.5	1.6	1.4	1.4
16:1	0.4	0.4	0.4	0.2	0.3
16:2	0.4	0.4	0.5	0.3	0.3
18:0	0.3	0.4	0.3	0.3	0.2
18:1	0.4	0.4	0.4	0.4	0.4
18:2	4.1	4.5	4.5	3.0	2.8
18:3	91.7	91.3	91.2	93.3	93.4
Total per cent unsaturated fatty-acid	97.0	97.0	97.0	97.2	97.2
DGDG					
14:0	1.0	1.0	0.9	1.0	1.0
16:0	11.1	11.2	11.6	12.6	12.4
16:1	0.4	0.4	0.4	0.4	0.4
16:2	0.2	0.4	0.4	0.3	0.3
18:0	1.0	1.3	1.3	1.2	1.1
18:1	0.6	0.7	0.9	0.6	0.6
18:2	4.0	3.8	4.2	3.3	3.2
18:3	81.7	81.2	80.3	80.6	81.0
Total per cent unsaturated fatty-acid	86.9	86.5	86.2	85.2	85.5

Table 41. (Continued). Changes in the percentage fatty-acid composition of sulpholipid in the non-hardened and hardened leaves of Hordeum vulgare during chilling at 5°C, 85 per cent RH

Fatty-acid	Control	Directly chilled		Hardened before chilling	
		chilled 12 hours	chilled 24 hours	chilled 12 hours	chilled 24 hours
SL					
14:0	7.0	4.5	7.1	6.3	6.3
16:0	24.1	23.6	24.4	22.9	23.9
16:1	2.1	5.0	3.8	2.1	1.8
16:2	1.9	4.6	3.3	2.4	2.3
18:0	2.2	3.6	2.1	2.3	2.3
18:1	1.8	3.2	1.8	1.7	1.8
18:2	5.2	6.0	5.4	4.4	3.3
18:3	55.7	49.5	52.1	57.9	57.3
Total per cent unsaturated fatty-acid	66.7	68.3	66.4	68.5	67.5

Table 42. Changes in the percentage fatty-acid composition of the total polar lipid fraction and weights of polar lipids in the non-hardened leaves of Gossypium hirsutum during chilling at 5°C, 85 per cent RH

Total fatty-acid composition of the polar lipids				Weights of polar lipids (per cent lipid composition by weight)			
Fatty-acid	Control	chilled 12 hours	chilled 24 hours	Lipid	Control	chilled 12 hours	chilled 24 hours
14:0	2.6	3.0	5.3	DGDG	17.4	17.1	20.4
16:0	13.6	13.7	17.2	SL	2.2	2.9	2.6
16:1	1.5	2.2	2.4	PE	9.8	8.9	10.6
16:2	1.5	2.0	2.8	PI	2.4	4.6	5.1
18:0	1.4	2.1	2.3	PA	2.6	3.5	7.2
18:1	5.9	5.7	5.7	PG	6.3	7.3	7.7
18:2	16.5	14.6	16.5	PC	24.6	19.7	19.7
18:3	57.0	56.7	47.8	MGDG	34.7	36.0	26.7
Total per cent unsaturated fatty-acid	82.4	81.2	75.2				

Table 43. Changes in the percentage fatty-acid composition of the total polar lipid fraction and weights of polar lipids in the non-hardened leaves of Phaseolus vulgaris during chilling at 5°C, 85 per cent RH

Total fatty-acid composition of the polar lipids				Weights of polar lipids (per cent lipid composition by weight)			
Fatty-acid	Control	chilled 12 hours	chilled 24 hours	Lipid	Control	chilled 12 hours	chilled 24 hours
14:0	2.2	2.1	3.0	DGDG	21.7	22.6	22.2
16:0	12.5	12.9	13.7	SL	1.7	2.0	2.0
16:1	1.8	2.2	2.6	PE	8.7	9.4	9.2
16:2	1.1	1.0	1.3	PI	3.0	3.6	3.6
18:0	2.6	2.7	2.8	PA	2.6	3.0	5.2
18:1	2.2	1.7	1.6	PG	9.5	9.8	11.6
18:2	10.8	10.4	10.0	PC	17.8	17.4	16.5
18:3	66.8	67.0	65.0	MGDG	35.0	32.2	29.7
Total per cent unsaturated fatty-acid	82.7	82.3	80.5				

B) Changes in the weights of the polar lipids of chill-sensitive and chill-resistant species on chilling at 5°C, 85 per cent RH

Chill-sensitive species

The chilling of non-hardened chill-sensitive plants caused rapid decreases in the weights of all phospho- and glycolipids (Figs. 16 and 17 respectively), except for PA which increased. The increase in PA suggests that loss of phospholipid was due to increased phospholipase activity as a result of the phase change.

Hardening the leaves of chill-sensitive species before chilling reduced subsequent losses of polar lipids so that the weight of all lipids was higher in the hardened and chilled plants than the non-hardened and chilled plants, except for PA (Figs. 16 and 17). When hardened Phaseolus vulgaris plants were chilled the weights of PC, PE and PI increased above the level at the end of the hardening period, indicating that the hardening treatment might involve the acquisition of the capacity for increased phospholipid synthesis on exposure to chilling, but similar increases did not occur on chilling hardened Gossypium hirsutum.

Chill-resistant species

No increase in the weight of the phospholipids of Hordeum vulgare leaves could be detected during 1 day at 5°C, the weight of phospholipid remaining constant (Fig. 16). The major changes in the weights of glycolipids on hardening Hordeum vulgare for 1 day at 5°C were small decreases in the weights of MGDG and DGDG in the plants grown at 12°C and a decrease in MGDG in the plants grown at 25°C before hardening at 5°C (Fig. 17).

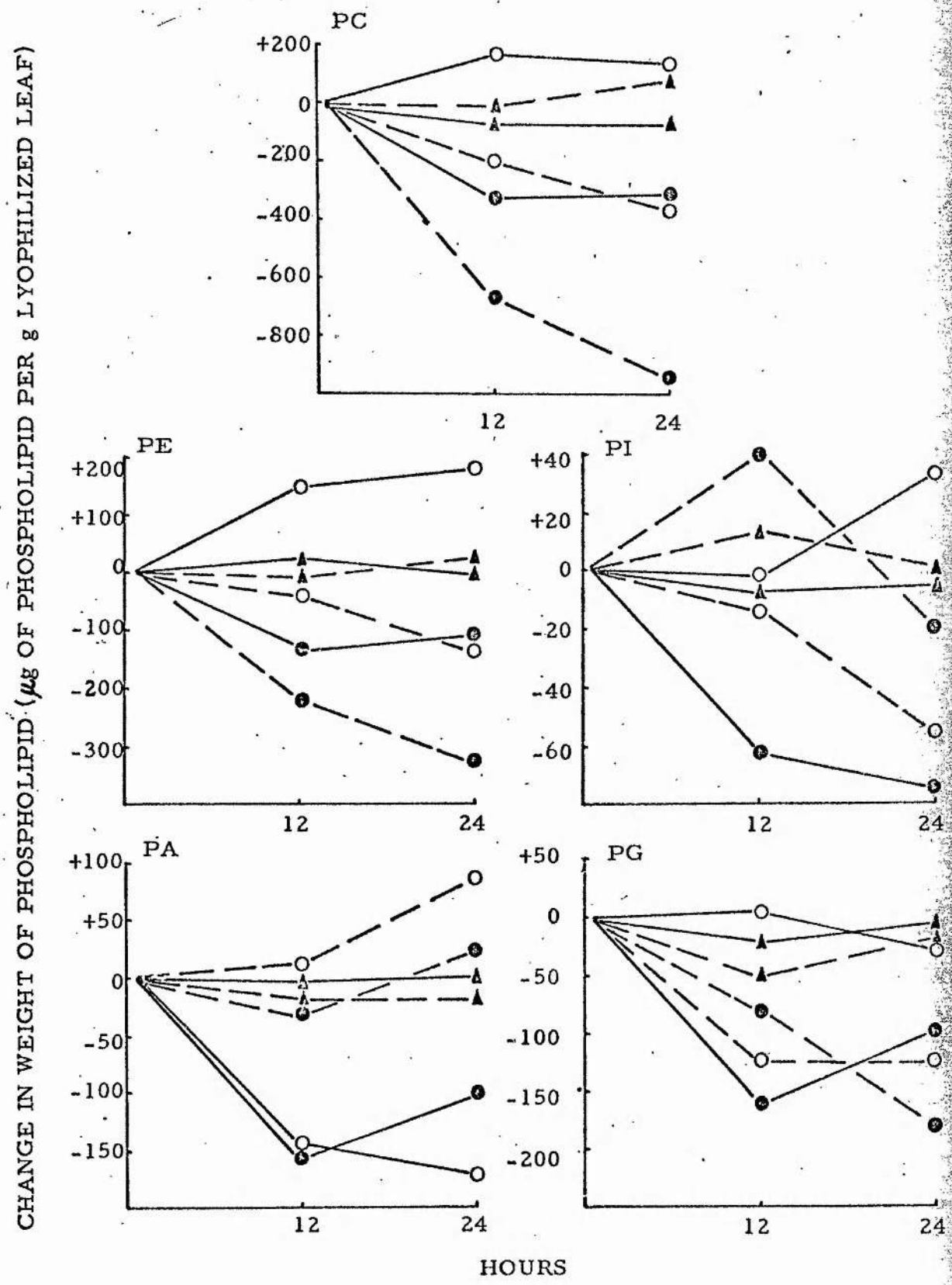


Fig. 16. Changes in weight of leaf phospholipids on chilling hardened and non-hardened plants at 5°C, 85 per cent RH. Continuous lines indicate plants which had been hardened for 4 days at 12°C, 95 per cent RH, before chilling, and broken lines the controls which remained at 25°C before chilling. ● Gossypium hirsutum; ○ Phaseolus vulgaris; ▲ Hordeum vulgare.

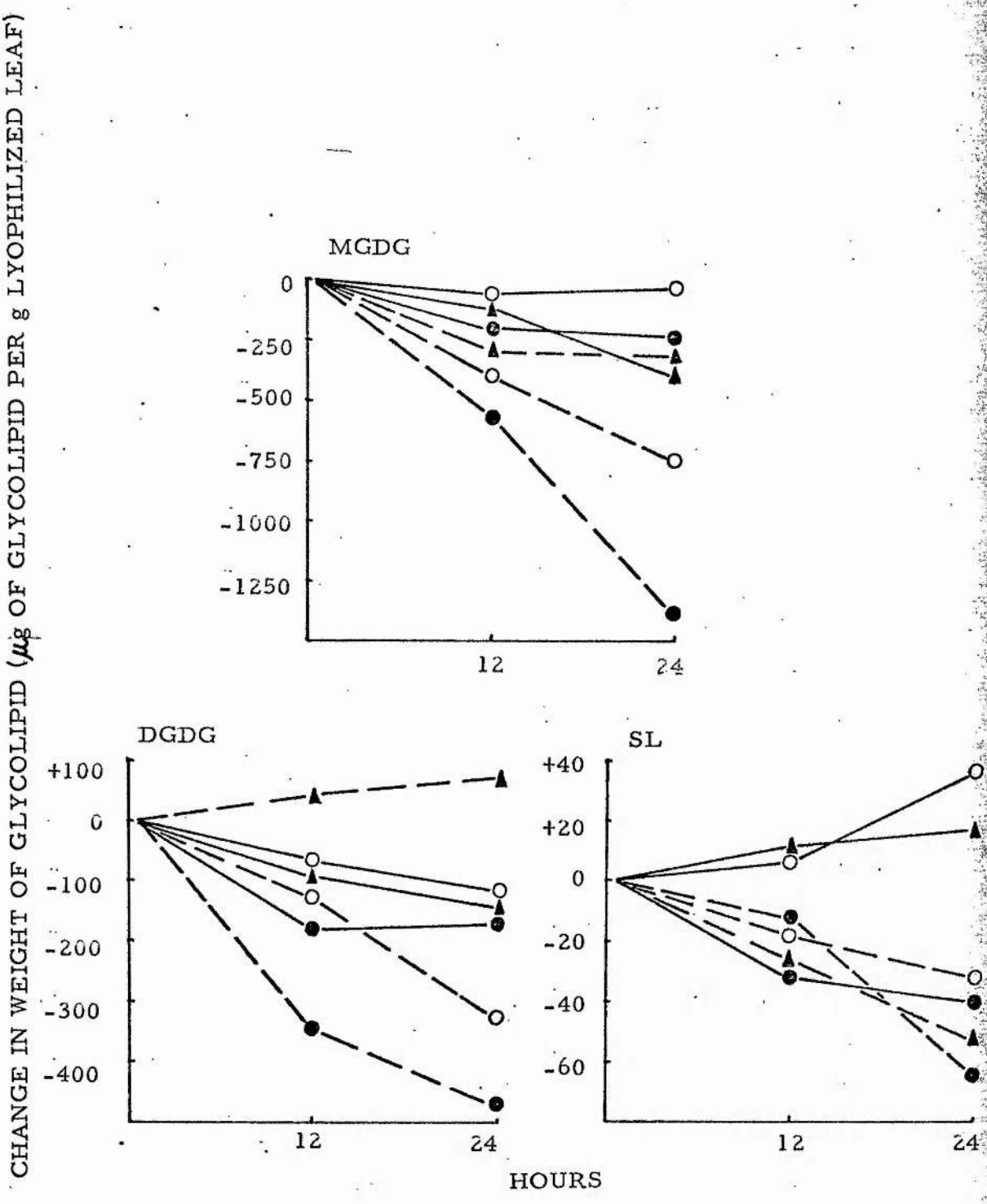


Fig. 17. Changes in the weight of leaf glycolipids on chilling hardened and non-hardened plants at 5°C, 85 per cent RH. Continuous lines indicate plants which had been hardened at 12°C, 95 per cent RH, before chilling, and broken lines controls which remained at 25°C before chilling. ● Gossypium hirsutum; ○ Phaseolus vulgaris; ▲ Hordeum vulgare.

4. THE EFFECTS OF CHILLING AT 5°C, 100 PER CENT RH AND GROWTH AT 12°C, 100 PER CENT RH.

A) Changes in the fatty-acid compositions of the polar lipids of chill-sensitive Phaseolus vulgaris on chilling at 5°C, 100 per cent RH and growth at 12°C, 100 per cent RH.

Leaves of Phaseolus vulgaris chilled at 100 per cent RH by enclosure in polythene bags for 4 days did not harden against chilling at 5°C, 85 per cent RH (P. 35). In addition, growth at 12°C, 100 per cent RH, for 4 days did not provide protection against chilling at 5°C, 85 per cent RH (P.35). In agreement with these results analyses of the leaf phospho- and glycolipids of plants transferred from 25 to 5°C, 100 per cent RH, and 12°C, 100 per cent RH, for 4 days showed no increase in the degree of unsaturation (Tables 44 to 51).

During the 4-day period at 5°C, 100 per cent RH, and 12°C, 100 per cent RH, the percentage of linoleic acid associated with the phospholipids decreased (Tables 47 to 51). This decrease in linoleic acid is similar to that which occurred in the controls maintained at 25°C, 100 per cent RH, over the 4-day period. The decreases in unsaturation with increase in physiological age at 25°C, 100 per cent RH, were similar to those at 25°C, 95 per cent RH, shown in Fig. 12. However, in plants maintained for 4 days at 5°C, 100 per cent RH, and 12°C, 100 per cent RH, the decrease in linoleic acid was usually accompanied by an increase in linolenic acid so that the total percentage unsaturated fatty-acid tended to remain constant or decrease (Tables 47 to 51). Therefore during 4 days' growth at 5°C, 100 per cent RH, and

12°C, 100 per cent RH, the percentage of linoleic acid associated with the phospholipids decreased. In contrast, during the effective hardening at 12°C, 95 per cent RH, the percentage of linoleic acid and total percentage unsaturated fatty-acid associated with the phospholipids increased (P. 130).

B) Changes in the weights of the polar lipids of chill-sensitive Phaseolus vulgaris on chilling at 5°C, 100 per cent RH and growth at 12°C, 100 per cent RH.

No significant changes were detected in the weights of glyco- and phospholipids during 4 days' growth at 5°C, 100 per cent RH, and 12°C, 100 per cent RH (Table 52). Therefore, chilling at 5°C, 100 per cent RH, prevented the rapid decreases in the weights of phospho- and glycolipids that accompany chilling-injury at 5°C, 85 per cent RH (Figs. 16 and 17).

Table 44. Changes in the percentage fatty-acid composition of monogalactosyl diglyceride of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	1.2	0.8	1.9	1.2
16:0	1.8	1.2	1.7	1.6
16:1	0.4	0.5	0.7	0.3
16:2	0.2	0.3	0.4	0.4
18:0	0.4	0.3	0.5	0.1
18:1	0.4	0.5	0.5	0.6
18:2	1.7	1.4	1.6	2.0
18:3	93.9	95.0	92.7	93.8
Total per cent 18:2 + 18:3	95.6	96.4	95.9	97.1
Total per cent unsaturated fatty-acid	96.6	97.7	94.3	95.8

Table 45. Changes in the percentage fatty-acid composition of digalactosyl diglyceride of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	1.2	1.4	1.0	0.8
16:0	9.1	9.1	9.8	8.6
16:1	0.6	0.8	1.2	0.6
16:2	0.4	0.4	1.0	0.8
18:0	2.0	2.0	1.0	0.8
18:1	0.7	0.7	1.2	0.9
18:2	1.6	1.5	2.0	1.7
18:3	84.4	84.1	82.8	85.8
Total per cent 18:2 + 18:3	86.0	87.5	88.2	87.5
Total per cent unsaturated fatty-acid	87.7	85.6	84.8	89.8

Table 46. Changes in the percentage fatty-acid composition of sulpholipid of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	4.9	4.4	5.2	4.8
16:0	21.7	23.8	21.4	23.1
16:1	2.3	4.1	3.8	3.8
16:2	3.2	2.8	3.1	3.1
18:0	4.8	3.0	3.5	3.7
18:1	2.2	2.0	2.1	2.2
18:2	3.0	3.0	2.8	1.5
18:3	57.9	56.9	58.1	57.8
Total per cent 18:2 + 18:3	60.9	59.9	60.9	59.3
Total per cent unsaturated fatty-acid	68.4	68.8	69.9	68.4

Table 47. Changes in the percentage fatty-acid composition of phosphatidyl choline of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	1.2	1.6	2.9	2.4
16:0	16.4	15.3	17.1	17.1
16:1	0.6	0.7	1.9	1.0
16:2	0.6	0.6	1.1	0.6
18:0	5.5	4.9	5.6	3.8
18:1	5.4	2.3	2.7	1.5
18:2	34.3	25.6	21.8	18.6
18:3	36.0	48.9	46.9	55.0
Total per cent 18:2 + 18:3	70.3	74.5	68.7	73.6
Total per cent unsaturated fatty-acid	76.9	78.1	74.4	76.7

Table 48. Changes in the percentage fatty-acid composition of phosphatidyl ethanolamine of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	3.8	4.1	5.4	5.1
16:0	27.2	27.2	32.2	27.7
16:1	2.0	1.7	2.9	1.8
16:2	1.2	0.7	1.4	1.4
18:0	3.7	3.8	4.2	3.8
18:1	2.6	1.6	2.3	1.5
18:2	28.0	28.0	22.0	21.7
18:3	31.5	32.9	29.6	36.9
Total per cent 18:2 + 18:3	59.5	60.9	51.6	58.6
Total per cent unsaturated fatty-acid	65.3	65.9	58.2	63.3

Table 49. Changes in the percentage fatty-acid composition of phosphatidyl inositol of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	4.3	4.3	7.7	5.7
16:0	31.3	29.9	24.9	33.1
16:1	1.7	1.8	3.3	4.4
16:2	2.4	2.0	2.7	2.4
18:0	5.4	5.3	5.7	5.4
18:1	2.5	4.2	2.6	2.6
18:2	15.7	15.2	11.9	9.3
18:3	36.7	37.3	41.2	37.1
Total per cent 18:2 + 18:3	52.4	52.5	53.1	46.4
Total per cent unsaturated fatty-acid	59.0	60.5	61.7	55.8

Table 50. Changes in the percentage fatty-acid composition of phosphatidic acid of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	10.1	11.2	12.6	13.2
16:0	13.0	12.8	17.6	14.6
16:1	2.5	2.4	1.8	2.2
16:2	2.7	2.6	1.3	1.6
18:0	4.8	5.3	3.7	7.4
18:1	6.4	4.8	1.8	4.2
18:2	29.0	29.7	21.6	26.5
18:3	31.5	31.2	39.4	30.3
Total per cent 18:2 + 18:3	60.5	60.9	61.0	56.8
Total per cent unsaturated fatty-acid	72.1	70.7	66.0	64.8

Table 51. Changes in the percentage fatty-acid composition of phosphatidyl glycerol of Phaseolus vulgaris leaves on chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity

Fatty-acid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
14:0	3.7	5.1	5.9	9.1
16:0	34.4	32.6	32.8	30.8
16:1	12.9	17.3	13.2	17.6
16:2	4.5	4.5	4.0	6.4
18:0	5.6	5.3	6.4	6.1
18:1	4.8	3.6	3.8	5.2
18:2	9.1	6.9	7.8	3.9
18:3	25.0	24.7	26.1	20.9
Total per cent 18:2 + 18:3	34.1	31.6	33.9	24.8
Total per cent unsaturated fatty-acid	56.3	57.0	54.9	54.0

Table 52. Changes in the weights of lipids in leaves of Phaseolus vulgaris during chilling at 5°C, 100 per cent relative humidity and growth at 12°C, 100 per cent relative humidity. Values expressed as mg of lipid per g lyophilized leaf.

Lipid	Control (Leaves 3 days old)	Chilled 48 hours at 5°C, 100 per cent relative humidity	Chilled 96 hours at 5°C, 100 per cent relative humidity	grown for 96 hours at 12°C, 100 per cent relative humidity
MGDG	10.55	11.30	10.90	10.12
DGDG	6.55	6.10	6.60	5.06
SL	0.60	0.45	0.60	0.40
PC	5.60	6.35	6.25	5.20
PE	2.70	3.00	2.90	3.14
PI	1.0	0.65	0.95	0.54
PG	3.25	2.95	3.05	2.00
PA	0.75	0.70	0.55	1.33
Total weight of polar lipid	31.10	31.50	31.80	27.79

DISCUSSION

1. A COMPARISON OF THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE AND CHILL-RESISTANT LEAVES GROWN AT 25°C

Previous studies by Lyons et al., (1964) have analysed the total fatty-acid composition of mitochondria isolated from chill-sensitive and chill-resistant fruits yet no investigations have been reported concerning the fatty-acid composition of individual membrane lipid components of leaves. The present investigation has shown that the degree of unsaturation of the glycolipids could not be related to the chill-sensitivity of the species and this is in agreement with the results of the analyses of the total leaf fatty-acid compositions of plants grown at 25°C (Chapter 3). In addition, the relationship between the total degree of unsaturation of the leaf phospholipids and chill-sensitivity was poor. In leaves, it was the total percentage of linoleic and linolenic acid associated with the phospholipids which showed the best relationship to chill-sensitivity. Phospholipids from chill-resistant Hordeum vulgare had, in general, a higher total percentage of linoleic and linolenic acid than chill-sensitive species, and the extremely chill-sensitive species Episcia reptans had, in general, a lower total percentage of these fatty-acids than the less chill-sensitive Gossypium hirsutum and Phaseolus vulgaris. Therefore, the total percentage of linoleic and linolenic acid of all the leaf phospholipids combined showed the most precise relationship to the chill-sensitivity of the species. Lyons and Asmundson (1965) have suggested that the relationship between the degree of unsaturation of the fatty-acids and chill-sensitivity is not precise due to the taxonomic

differences between species.

In contrast to leaves, the chill-sensitivity to mitochondria isolated from fruits is best related to the total percentage of unsaturated fatty-acid and not the total percentage of linoleic and linolenic acid, since the percentage of oleic acid was high in cauliflower buds and turnip roots. However, analyses of the fatty-acid compositions of individual phospholipids from fruit mitochondria might be expected to reveal a relationship similar to that shown above for leaf phospholipids. The results of Lyons et al., (1964) appear fortuitous when it is remembered that only 54 per cent of the total lipids of plant mitochondria are phospholipids (Schwertner and Biale, 1973). Because glycolipids are present in membranes, analyses of the total fatty-acid composition of most membranes might be expected to reveal no relationship between the degree of unsaturation and chill-sensitivity as indicated by the analyses of total leaf fatty-acid (Chapter 3).

2. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES DURING HARDENING AT 12°C, 95 PER CENT RH

Temperature acclimatization affords a unique opportunity to elucidate the biochemical mechanisms which enable plants to survive temperature stress. An analysis of the total leaf and chloroplast fatty-acids on hardening 4 chill-sensitive species showed only a small increase (approximately 3 per cent) in the percentage of linoleic acid (Chapter 3). Although this investigation used a total leaf fatty-acid extract, it was estimated that 60 per cent of the total leaf fatty-acids were associated with the polar lipids, which are thought to occur

almost exclusively within membranes (Kates, 1970). However, the significant relationship between the total percentage of linoleic and linolenic acid associated with the phospholipids and the chill-sensitivity of plants grown at 25°C (P.112) led to the hypothesis that the small increase in linoleic acid detected in the total leaf fatty-acid analyses on hardening was attributable to larger increases in unsaturation of specific phospholipids. Increases in unsaturation of the phospholipids on hardening may only just be detectable in total leaf fatty-acid analyses as the phospholipids comprise only 22 per cent of the total leaf lipids and 10 per cent of the chloroplast lipids (Table 3).

The results of the present chapter show that hardening chill-sensitive Gossypium hirsutum and Phaseolus vulgaris produced large increases in unsaturation of all the phospholipids analysed. Therefore, chill-resistance appears to be related to a requirement for a specific degree of unsaturation of the membrane phospholipids and not to the overall fatty-acid composition of the lipid matrix.

The increases in unsaturation of the phospholipids of Hordeum vulgare at 12°C were small in comparison to the increases in the degree of unsaturation of the phospholipids of chill-sensitive species at 12°C. This indicates that the increases in unsaturation during the hardening of chill-sensitive species, are not due to an indirect effect of temperature on fatty-acid metabolism unassociated with the development of chilling-resistance. Increases in unsaturation of the phospholipids of Gossypium hirsutum and Phaseolus vulgaris were also shown to be directly related to hardening as no increase

in unsaturation of the phospholipids of Episcia reptans occurred during growth of this species over 4 days at 15°C, the lowest temperature at which this species will grow without damage. In addition, no increase in the degree of unsaturation of the phospholipids occurred during the ineffective hardening of Phaseolus vulgaris on transfer from 25 to 12°C, 100 per cent RH. If temperature had an indirect effect on desaturase activity in chill-sensitive species, we would expect increases in the degree of unsaturation of both linoleic and linolenic acids, since it could be predicted that the enzyme which converts oleic acid to linoleic acid would be intimately involved in the conversion of linoleic acid to linolenic acid (Kleinschmidt and McMahon, 1970). Furthermore, Lyons and Asmundson (1965) have shown from studies on the solidification points of mixtures of pure fatty-acids, that the most rapid manner by which a plant could lower the transition temperature would be to increase the percentage of linoleic acid (since linoleic acid has the same effect as linolenic acid in lowering the temperature of the phase change). This theoretical prediction is now seen in this present investigation to be correct. Therefore increases in unsaturation of the phospholipids of Gossypium hirsutum and Phaseolus vulgaris on hardening are not due to an indirect effect of temperature on fatty-acid metabolism. The results suggest there is a control mechanism in the cell which increases the degree of unsaturation of the phospholipids of Gossypium hirsutum and Phaseolus vulgaris on hardening, thereby lowering the temperature of the phase change in the lipid layer of the cellular membranes.

Hardening was found to require 2 to 4 days at a temperature slightly above the chilling range and it is thought that this time-period may reflect the turnover or synthesis rate of the linoleic acid moiety of the phospholipids. Turnover rates of lipids of potato and cauliflower cells have been shown to vary between 3 days to 3 months (Mazliak, 1973). In Episcia reptans the rate of turnover of the membrane lipids and synthesis of linoleic acid may be slower than in species which harden quickly, thereby limiting the speed at which hardening occurs. Alternatively, a more plausible explanation for the inability of Episcia reptans to harden quickly is that the activity or quantity of desaturases is not increased or induced at the hardening temperature, so that no increase in the degree of unsaturation of the phospholipids occurs over the 4-day hardening period. The inability of Episcia reptans to harden over 4 days at 15°C is discussed in detail on page 193.

The fatty-acid composition of the phospholipids varies between different species (Tables 23 to 27) and perhaps within the same species depending on the membrane system from which they are isolated. Because we analysed total leaf phospholipid it is not known if the degree of unsaturation of the phospholipids increased in all membranes during hardening. Nevertheless, the following results suggest that increases in unsaturation of the phospholipids occur in the majority of the cellular membranes on hardening. Phase changes have been detected at approximately the same temperature of 10-12°C (Raison, 1973) in membrane preparations

which differed widely in total fatty-acid composition (i.e. chloroplasts and mitochondria) indicating that increases in unsaturation of the phospholipids must occur in both these membrane systems on hardening if the phase change is to be prevented. This theory is supported by the small increase in the percentage of linoleic acid in the total fatty-acid analyses of the chloroplasts of chill-sensitive species on hardening (Table 16), which is probably attributable to an increase in linoleic acid associated with the phospholipids. Increases in unsaturation of the phospholipids of the plasmallema are implied by the reduction in water loss from hardened leaves on chilling (Fig. 1). Finally, if increases in unsaturation occurred in only a small fraction of the total cellular membranes, they would not be detectable by the analysis of total leaf phospholipid, unless the increase was very large. Therefore, it is concluded that increases in unsaturation occur in the majority of the membrane systems of the cell on hardening thus lowering the transition temperature of the membrane lipids. Phase changes are not solely confined to mitochondrial membranes as is implied from studies of mitochondria isolated from chill-sensitive fruits.

3. CHANGES IN THE FATTY-ACID COMPOSITIONS AND WEIGHTS OF THE POLAR LIPIDS OF CHILL-RESISTANT HORDEUM VULGARE DURING HARDENING AT 5 AND 12°C AGAINST FREEZING-INJURY AT -4°C

A fundamental metabolic difference between chill-sensitive and chill-resistant species is that chill-resistant plants are able to respond to a decrease in temperature to 5°C by increasing the degree of unsaturation and weight of most phospholipids. In contrast, this temperature is too low for the hardening of many chill-sensitive

species and the degree of unsaturation and weight of phospholipids decreases, reflecting chill-injury.

Hardening Hordeum vulgare for 4 days at 12°C was less effective in preventing freezing-injury than 1 day at 5°C. This result correlates well with changes in the fatty-acid composition during hardening at the two temperatures. At 12°C no increase in the percentage of linoleic acid associated with the phospholipids was detectable, the main change being a small increase in total per cent unsaturated fatty-acid in PC, PE and PA after 4 days. Increases in the percentage of linoleic acid and total per cent unsaturated fatty-acid were, however, detectable in PC, PE and PI after only 1 day at 5°C. The less effective hardening at 12°C is in agreement with the results of Harvey (1922) who found that hardening against freezing-injury did not generally occur above 5 to 10°C. Increases in unsaturation of the fatty-acids, similar to those which occurred in Hordeum vulgare at 12 and 5°C, have also been detected on hardening alfalfa roots to sub-zero temperatures (Gerloff et al., 1966; Grenier et al., 1973).

There was no detectable increase in weight of phospholipid during hardening for 1 day at 5°C, although the weights of PC, PE and PG increased over 4 days at 12°C. Similarly, Rodniov et al., (1973) detected small increases in the weights of PC and PG on hardening potato leaves against frost-injury. Increases in the weights of phospholipid on hardening plants to sub-zero temperatures may be indicative of increases in unsaturation and fluidity of the membranes

or folding of the plasmallema and replication of membrane organelles (Siminovitch et al., 1968). Decreases in the weights of the glycolipids MGDG and DGDG in Hordeum vulgare at 5°C suggest that these lipids may be catabolised either as a source of energy or fatty-acids for the increases in unsaturation and weight of the phospholipids on hardening, although no change was detectable in the weights of the glycolipids at 12°C. Rodniov et al., (1973) also detected a decrease in the weights of glycolipids on hardening potato leaves against frost-injury. The manner in which these changes in phospholipid composition may protect Hordeum vulgare against frost-injury are discussed on P. 194.

4. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES ON CHILLING AT 5°C, 85 PER CENT RH

Chilling Gossypium hirsutum and Phaseolus vulgaris resulted in small decreases in the percentage of linoleic acid (18:2) associated with all the phospholipids, except for PG. No decrease in the percentage of linolenic acid (18:3) of any phospholipid was detected on chilling chill-sensitive plants as would be expected from the decrease in the percentage of linolenic acid of the total leaf fatty-acid on chilling (Chapter 3). In this chapter it was mentioned (P. 91) that the decrease in the percentage of linolenic acid in the total leaf fatty-acid analyses might raise the temperature at which the phase change would reverse when the plant was returned to the warmth and that this elevated temperature might be too high for the growth of the plant so that the phase change would not be reversible. The decrease in

linoleic acid of the phospholipids on chilling may also raise the temperature at which the phase change will reverse, although the changes detected were very small. Because the degree of unsaturation of the phospholipids has been shown to be directly related to the chill-sensitivity of the species and no decrease in linolenic acid of the phospholipids occurred on chilling, it is considered that the decreases in linolenic acid of the total leaf fatty-acid analyses on chilling (Chapter 3) may not be important in determining the temperature at which the phase change reverses on return to the warmth. The rapid decreases in linolenic acid of the total leaf fatty-acid analyses may, however, be due to autoxidation which may result in membrane damage.

It would be expected that those lipids with a high percentage of linolenic acid such as MGDG would be more susceptible to autoxidation of this acid than those lipids with a lower percentage of this fatty-acid. The decrease in the percentage of linolenic acid associated with MGDG, DGDG and SL of Gossypium hirsutum on chilling suggests that the linolenic acid associated with these lipids may be more susceptible to autoxidation than that associated with the phospholipids (Table 39). Hardening Gossypium hirsutum before chilling reduced this decrease in linolenic acid associated with the glycolipids (Table 39). Therefore, the slower decrease in the percentage of linolenic acid in the total leaf fatty-acid analyses of the hardened plants of Cucumis sativus and Saintpaulia grandiflora on chilling, reported in Chapter 3, may be due, in part, to a decreased rate of autoxidation of linolenic acid associated with the glycolipids. There was no decrease in the percentage of linolenic acid associated with the glycolipids of Phaseolus vulgaris on chilling (Table 40) and this correlates well with the very small decrease in the percentage of linolenic acid in the total leaf fatty-acid analyses on chilling (Fig. 5).

A decrease in the percentage of linolenic acid in the total leaf fatty-acid analyses on chilling could also be caused by a decrease in the weight of particular lipids, especially those predominantly esterified with linolenic acid. Table 42 showed that the decrease in the percentage of linolenic acid in the total fatty-acid composition of the polar lipids of Gossypium hirsutum on chilling was due to a more pronounced decrease in the weights of MGDG and PC than in other lipids, as well as a decrease in the percentage of linolenic acid associated with the glycolipids (Table 39). In Phaseolus vulgaris the decrease in the percentage of linolenic acid was small in both the total leaf fatty-acid and total polar lipid analyses. Therefore changes in unsaturation of the total leaf fatty-acid may be due, in part, to changes in total polar fatty-acid composition on chilling. Summarizing briefly, the slower decrease in the percentage of linolenic acid in the leaves of hardened Cucumis sativus and Saintpaulia grandiflora on chilling (Fig. 9, Chapter 3) is considered to be due to a reduction in the weight loss of specific lipids (e.g. MGDG and PC) and perhaps a reduction in autoxidation of linolenic acid associated with the glycolipids.

5. CHANGES IN THE WEIGHTS OF THE POLAR LIPIDS OF CHILL-SENSITIVE SPECIES ON CHILLING AT 5°C, 85 PER CENT RH

Chilling the leaves of non-hardened chill-sensitive species resulted in rapid decreases in the weights of phospho- and glycolipids, except for PA which increased. These changes are very similar to the rapid decreases in the weights of lipids in potato leaves due to frost-injury (Rodniov et al., 1973). In contrast to leaves, the decline in phospholipid of chill-sensitive fruits on chilling occurs slowly, the decline in phospholipid content of sweet potato mitochondria only beginning after 4 days at the

chilling temperature (Yamaki and Uritani, 1972). Small decreases in the weight of phospholipid and unsaturation occurred on chilling hardened leaves of Gossypium hirsutum and Phaseolus vulgaris reflecting that hardening was not completely effective in preventing the loss of membrane phospholipid.

The increase in PA on chilling Gossypium hirsutum and Phaseolus vulgaris indicates that the decline in phospholipid was due to increased phospholipase activity as a result of the phase change. In relation to both chilling and freezing-injury it is thought that hydrolysis of lipids may be due to the action of phospholipase D or galactolipase enzymes. Phospholipase D was found to occur in a wide variety of leaves by Quarles and Dawson (1969) and galactolipase has been detected in leaves by Sastry and Kates (1964).

Wright (1971) suggested that the decrease in weight of phospholipid in the leaves of Cucumis sativus on chilling was dependent on water loss since the weight of phospholipid remained unchanged when plants were chilled at 100 per cent RH by enclosure in polythene bags. When the leaves were chilled at 85 per cent RH their fresh weight decreased by 38 to 55 per cent and the weight of phospholipid decreased rapidly. He suggested that dehydration of the cells may eventually lead to the entry of air into the cell, deranging cell compartmentation and exposing lipids which are otherwise protected from the action of enzymes such as phospholipase D and galactolipase. However, on P. 35 it was shown that plants of Episcia reptans chilled at 100 per cent RH were severely chill-injured, although the rate of development of injury was slower.

Therefore water loss on chilling is not essential for a decrease in the weight of phospholipid to occur in all species. Changes in the permeability properties of the membranes of Episcia reptans to substrates and enzymes as a result of the phase change in the lipid layer may render them more susceptible to attack by phospholipase and galactolipase enzymes and cause imbalances in metabolism resulting in injury. In contrast, leaves of Phaseolus vulgaris are able to withstand these permeability changes and imbalances in metabolism when chilled at 100 per cent RH, so that no injury occurs. Nevertheless, a reduction in the amount of water lost from the leaf is important in preventing chilling-injury in both Episcia reptans and Phaseolus vulgaris, as chilling Episcia reptans at 5°C, 100 per cent RH, retarded the development of injury (P. 35). Activation of galactolipase and phospholipase by low temperatures in Episcia reptans is another possibility that cannot be excluded.

6. CHANGES IN THE FATTY-ACID COMPOSITIONS OF THE POLAR LIPIDS OF CHILL-SENSITIVE PHASEOLUS VULGARIS ON CHILLING AT 5°C, 100 PER CENT RH AND GROWTH AT 12°C, 100 PER CENT RH.

The degree of unsaturation of the phospholipids of Phaseolus vulgaris leaves grown at 5°C, 100 per cent RH and 12°C, 100 per cent RH for 4 days did not increase. In agreement with these results the leaves did not harden and were as susceptible to chilling-injury at 5°C, 85 per cent RH, as leaves transferred directly from 25 to 5°C, 85 per cent RH. These results provide additional evidence to that reported on page 171 that increases in unsaturation of the phospholipid fatty-acids of Gossypium hirsutum and Phaseolus vulgaris leaves during the effective hardening at 12°C, 95 per cent RH, are not due to

an indirect effect of temperature on lipid metabolism, unassociated with the development of chill-resistance.

Desaturase activity

We must now consider why no hardening is achieved in Phaseolus vulgaris during 4 days growth at 5°C, 100 per cent RH, and 12°C, 100 per cent RH. Unsaturated fatty-acids are produced in leaves by a sequential series of dehydrogenations of the saturated palmitic and stearic acids (Harris and James, 1969 a). The major cofactors involved in desaturation reactions are oxygen, NADPH or NADH, CoA and acyl-carrier protein and the chloroplasts are the main site of fatty-acid synthesis in the leaf. Harris and James (1969 a, b) have demonstrated that in non-photosynthetic tissues (e. g. seeds) oxygen appears to be the main rate limiting factor of desaturation of fatty-acids. In very active photosynthetic tissues such as leaves oxygen could not be demonstrated to be rate limiting for fatty-acid desaturations in the light because of excess oxygen produced within the cell by photosynthesis; only in the dark could an effect of increased supply of oxygen on the formation of unsaturated fatty-acids in photosynthetic tissue be demonstrated. During the effective hardening of chill-sensitive plants at 12°C, 95 per cent RH, in the light, it is predicted that the cofactors oxygen and NADPH will be available for desaturase activity due to photosynthetic production. However, when plants are enclosed in polythene bags at 12 and 5°C the concentration of carbon dioxide inside the bag will be rapidly diminished by photosynthetic fixation. This decrease in carbon dioxide concentration will result in the cessation of photosynthesis and hence the photosynthetic production of oxygen and NADPH, essential for desaturase activity. In support of this theory Harris, James and

Harris (1967) found that the formation of unsaturated fatty-acid in leaf tissue was suppressed by anaerobiosis in the light. The respiration of the plants enclosed in the polythene bags may, however, release some carbon dioxide for photosynthesis, but the amount released is probably small at low temperatures and may be rapidly absorbed by the condensed moisture on the inside of the polythene bag. Therefore, it is considered that hardening did not occur over a 4-day period at 12°C, 100 per cent RH, and 5°C, 100 per cent RH, by enclosure in polythene bags, due to the rapid utilization of carbon dioxide inside the bag. This is thought to have resulted in the cessation of photosynthesis and a decrease in the supply of oxygen and NADPH, which are essential cofactors for desaturase activity and an increase in unsaturation of the phospholipids during hardening.

Hardening in the dark

At this point it is appropriate to consider why leaves of Phaseolus vulgaris held for 2 days at 12°C, 95 per cent RH, in the dark did not harden against chilling-injury at 5°C, 85 per cent RH (P. 32). Harris and James (1969 a) have shown that the absence of light diminishes the biosynthesis of all the fatty-acids and shows some inhibition of unsaturated fatty-acid synthesis in Chlorella vulgaris. Therefore, it is suggested that growing leaves of Gossypium hirsutum and Phaseolus vulgaris at 12°C, 95 per cent RH, in the dark for 2 days is not effective against subsequent chilling at 5°C, 85 per cent RH, due to the cessation of photosynthesis and a decrease in the availability of oxygen and NADPH for desaturase activity. Thus the absence of light at 12°C, 95 per cent RH, limits desaturase activity to a low level and prevents

an increase in unsaturation of the phospholipids which is essential for hardening to be effective.

7. CHANGES IN THE FATTY-ACID COMPOSITIONS AND WEIGHTS OF THE POLAR LIPIDS DURING THE GROWTH OF CHILL-SENSITIVE AND CHILL-RESISTANT PLANTS OVER 4 DAYS AT 25°C

An increase in physiological age over a 4-day period at 25°C resulted in decreases in the weight of phospholipid and the percentage of linoleic acid associated with the phospholipids of chill-sensitive and chill-resistant plants. Maturation of leaves over long periods (up to 200 days) has been shown to involve a decrease in the percentage of linolenic acid associated with the phospholipids from 30 to 10 per cent and an increase in the percentage of linoleic acid of approximately 4 per cent (Klopfenstein and Shigley, 1967). Because changes in the fatty-acid composition of the phospholipids over 4 days at 25°C occur at a much faster rate and are essentially opposite to those detected by other workers during maturation over long periods, it may be considered that the decrease in linoleic acid over 4 days at 25°C is related to adaptation to growth at constant high temperature by producing a more saturated fatty-acid composition. However, the degree of unsaturation of the phospholipids of Phaseolus vulgaris during 4 days growth at 5°C, 100 per cent RH and 12°C, 100 per cent RH, decreased in a similar manner to the controls held at 25°C. Therefore, the decrease in unsaturation of the phospholipids over the 4-day period is probably related solely to an increase in physiological age of the leaf and not to the adaptation of the plants to growth at constant high temperature.

The decrease in unsaturation of the phospholipids of chill-sensitive species with increase in physiological age at 25°C is probably the cause of the less effective hardening of older leaves (P. 38). These changes in unsaturation and ability to harden with increase in age have important implications for the survival of young plants. It appears that leaves of young plants are better equipped to withstand low temperature stress at a critical stage in their establishment when many factors can determine the success or failure of a species in its environment.

SUMMARY

The chill-sensitivity of leaves grown at 25°C was best related to the total percentage of linoleic and linolenic acid associated with each phospholipid and not the total degree of unsaturation of each phospholipid. Therefore a comparison of the total percentage of linoleic and linolenic acid of all the phospholipids combined showed the most marked relationship to the chill-sensitivity of the species. The degree of unsaturation of the glycolipids could not be related to the chill-sensitivity of the species and this is in agreement with the results of the analyses of the total leaf fatty-acid of plants grown at 25°C (Chapter 3).

An examination was also made of the effects of low temperature acclimatization on the fatty-acid composition of the polar lipids of two chill-sensitive species, Gossypium hirsutum and Phaseolus vulgaris, which harden at 12°C, 95 per cent RH, against chilling-injury at 5°C, 85 per cent RH. As controls, the effect of hardening temperatures on a chill-resistant species, Hordeum vulgare, and a chill-sensitive species, Episcia reptans, which did not harden, was investigated. It was found that the degree of unsaturation of the fatty-acids associated with all the phospholipids increased during hardening of Gossypium hirsutum and Phaseolus vulgaris. These increases were shown to be positively related to the increased tolerance of the species to chilling-injury as similar increases did not occur during the growth of Hordeum vulgare at 12°C and the ineffective attempts at hardening Episcia reptans at 15°C over 4 days, the lowest temperature this species can withstand without injury. The effective hardening of Gossypium hirsutum and Phaseolus vulgaris produced no change in the fatty-acid composition

of the glycolipids of these plants and this accorded with earlier observations which showed no increase in unsaturation of the total leaf fatty-acid on hardening (Chapter 3). Therefore, in leaves, it was the phospholipid fraction alone and representing only 22 per cent of the total leaf fatty-acids that had its degree of unsaturation positively related to the chilling tolerance of the species investigated.

Chilling-injury in Phaseolus vulgaris leaves at 5°C could be prevented by enclosing the plants in polythene bags, thus maintaining 100 per cent RH. Plants held at this temperature and humidity for 4 days did not harden against subsequent chilling at 5°C, 85 per cent RH. In agreement with this finding no increase in unsaturation of the phospholipids was detected over 4 days growth at 5°C, 100 per cent RH. In addition, growth for 4 days at 12°C, 100 per cent RH, was not effective against subsequent chilling at 5°C, 85 per cent RH, and resulted in no increase in unsaturation of the phospholipids. It is suggested that plants grown at 5 and 12°C, by enclosure in polythene bags, do not harden against subsequent chilling-injury at 5°C, 85 per cent RH, because the level of carbon dioxide within the bag decreases rapidly causing the cessation of photosynthesis, which restricts desaturase activity by reducing the availability of the cofactors oxygen and NADPH, essential for desaturase activity. Similarly, it is concluded that hardening does not occur in the dark at 12°C, 95 per cent RH, due to the ending of photosynthesis which restricts desaturase activity due to a decreased availability of oxygen and NADPH.

The degree of unsaturation and weight of phospholipids decreased in the leaves of the chill-sensitive species with increase in physiological age at 25°C and this was related to the increased susceptibility of older leaves to chilling-injury and the less effective hardening of older leaves.

Hardening chill-resistant Hordeum vulgare for 1 day at 5°C, or 4 days at 12°C, reduced the susceptibility of the leaves to frost-injury at -4°C. Older leaves of this species maintained at 25°C were more susceptible to frost-injury than younger leaves. These changes in susceptibility to frost-injury could be related to alterations in both the weight and degree of unsaturation of the phospholipids.

FINAL DISCUSSION

Chilling-injury to plants depends not only on the chilling conditions (P. 18) but also on the temperature of growth before chilling. Extremely chill-sensitive species (e.g. Episcia reptans, category 1, P. 21) cannot be readily hardened against chilling-injury. In contrast, less chill-sensitive species (e.g. Phaseolus vulgaris, category 2, P. 22) can be readily hardened against chilling-injury at 5°C, 85 per cent RH, by 4 days growth at 12°C, 95 per cent RH, before chilling. Chilling-injury therefore poses 4 main questions:-

1. What is the mechanism of chilling-injury in leaves?
2. How does the hardening process prevent chilling-injury?
3. Why are extremely chill-sensitive species (e.g. Episcia reptans) incapable of rapid hardening?
4. Is there a unifying role for the increases in unsaturation of the phospholipids during hardening against chilling and freezing-injury which affords protection against both types of damage?

1. What is the mechanism of chilling-injury in leaves?

From studies of mitochondria isolated from the fruits and storage organs of plants there is general agreement that an immediate and direct effect of a decrease in temperature to approximately 10°C in chill-sensitive tissues is a phase change in the lipid layer of the cellular membranes from a liquid-crystalline to a solid gel. Fig. 18 presents in schematic fashion the consequences of this phase change in the cellular membranes of chill-sensitive leaves. As the temperature is lowered the membrane lipids solidify at the critical temperature (usually 10 to 12°C) and the change in state may cause contraction of the lipids resulting in the appearance of 'holes' or 'cracks' leading to increased membrane permeability. In addition, Trauble and Haynes (1971) have suggested that an increase in permeability may also accompany the phase transition due to changes in membrane thickness, the structure of the hydrocarbon chains and the arrangement of the polar head groups (for further details see P. 5). This increased permeability accounts for the rapid water loss (P. 27) and ion leakage (Wright and Simon, 1973) that occurs on chilling leaves. The phase transition also increases the E_a of membrane bound enzymes (Lyons, 1972) leading to a suppressed reaction rate and may establish an imbalance with non-membrane bound systems such as glycolysis. The transitory increase in the concentration of ethanol in the leaves of chill-sensitive species on chilling (Chapter 2) is considered to be due to the increased E_a of the enzymes bound to the mitochondrial membranes. Suppressed mitochondrial respiration as a result of the phase change may lead to reduced ATP supply (Stewart and Guinn, 1969) and cessation of

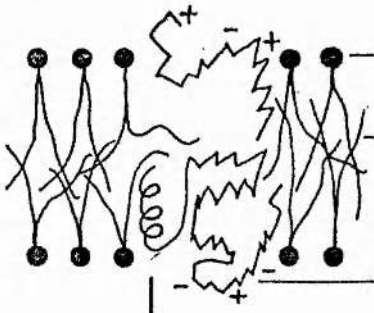
protoplasmic streaming (Lewis, 1956). Similar events probably occur in the chloroplast where the phase transition leads to suppressed activity (Shneyour et al., 1973) and changes in the levels of metabolites after brief chilling (Taylor, Jepsen and Christeller, 1972).

It is suggested that metabolic imbalances produced by the increase in E_a of membrane bound enzymes is the main pathway leading to injury of species such as Episcia reptans (category 1). Water loss on chilling these species may be of secondary importance since injury could not be prevented by maintaining 100 per cent RH at 5°C. In contrast, the main pathway leading to chilling-injury in species such as Phaseolus vulgaris (category 2) may be water loss. Metabolic imbalances in the leaves of these species may be of secondary importance since injury could be prevented by maintaining 100 per cent RH at 5°C.

The length of time a species can be chilled without incurring injury depends on the species used, the chilling temperature, the temperature of growth before chilling, age of leaf, humidity of the air, light intensity, nutrition of the plant and growth conditions on return to the warmth (P. 18). If no degenerative injury occurs during chilling, the phase change in the lipid portion of the membranes is completely reversible. Thus with a short chilling period followed by return to 25°C, the respiration rate increases transiently and normal metabolism is quickly re-established (Chapter 2). If chilling is prolonged degenerative changes occur and the respiration

Fig.18. A schematic pathway of the events leading to chilling-injury in leaves. (Membrane model proposed by Singer, 1971).

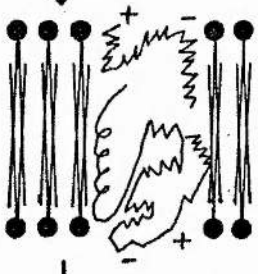
(The + and - signs indicate the ionic residues of the proteins)



LIQUID-CRYSTALLINE

Chilling temp. 5°C

Phase transition



SOLID GEL

Increase in E_a of membrane-bound enzymes

Increased membrane permeability

Imbalance of metabolism

Water loss and ion leakage

Accumulation of metabolites e.g. Ethanol. Reduction in ATP. Cessation of protoplasmic streaming

Brief exposure and return to 25°C

Limit of tolerance to chilling depending on species used, temperature of growth before chilling, age of leaf, humidity of air, light intensity and nutrition of the plant.

PROLONGED CHILLING

Loss of cellular compartmentation due to increased membrane permeability and dehydration.

Breakdown of the cellular membranes due to phospholipase and galactolipase activity and lipid peroxidation.

Loss of reversibility of the phase change.

INJURY AND DEATH OF THE LEAVES

rate decreases on return to 25°C reflecting cell death (Chapter 2). Chilling eventually results in the loss of cellular compartmentation and organisation, the breakdown of the cellular membranes due to phospholipase and galactolipase activity and peroxidation, the loss of the reversibility of the phase change and ultimately the injury and death of the leaves.

Therefore the primary response in chilling-injury is a temperature induced phase transition in the membrane lipids of the cellular membranes.

2. How does the hardening process prevent chilling-injury?

The acclimatization of animals to low temperatures and plants to sub-zero temperatures is known to be associated with increases in the degree of unsaturation of the fatty-acids contained in the cellular membranes (Table 2). Until the present study, little evidence existed for a similar relationship between plant membranes and the ability of thermophilic plants to adapt to chilling temperatures, as most studies on chilling-injury have centered on tissues which do not really harden, namely fruits and storage organs. Hardening chill-sensitive leaves of Phaseolus vulgaris and Gossypium hirsutum at 12°C, 95 per cent RH, against chilling-injury at 5°C, 85 per cent RH, was shown to be specifically related to the degree of unsaturation of the phospholipids, which are only minor components of some membranes (e.g. chloroplasts, P. 11), in terms of total fatty-acid composition. Increases in unsaturation of the phospholipids on hardening chill-sensitive leaves at 12°C are considered to lower the transition temperature of the lipid layer of the cellular membranes,

thus preventing a phase change from a liquid-crystalline to a solid gel at chilling temperatures. Therefore, in the hardened chill-sensitive plants the increase in membrane permeability and increase in E_a of membrane bound enzymes which accompany the phase change are prevented on chilling.

Induction of desaturase activity

The desaturation of fatty-acids in non-photosynthetic tissues (e. g. seeds) seems to be regulated by the concentration of oxygen in the aqueous phase (Harris and James, 1969 a, b). As the temperature falls lipids of lower melting point are considered to be supplied automatically to non-photosynthetic tissues by the increased solubility of oxygen in water. No elaborate controls are thus required and the system is self regulated. In contrast to non-photosynthetic tissues the concentration of oxygen in the illuminated leaf cannot be shown to be rate limiting for the synthesis of unsaturated fatty-acids, due to the production of excess oxygen in the leaf by photosynthesis (Harris and James, 1969 a). Therefore, the degree of unsaturation of the phospholipids of plant leaves is probably determined by the selective synthesis or activation of desaturases at the environmental temperature. Plant fatty-acid composition is presumably also under genetic control. Thus the relative amounts of saturated and unsaturated fatty-acid which accumulate in leaves are probably determined by a combination of these effects. For hardening to occur in Gossypium hirsutum and Phaseolus vulgaris it is considered that the activity or quantity of desaturases may be increased or induced at the hardening temperature, resulting in an increase in the degree of unsaturation of the phospholipids. In addition,

an adequate supply of cofactors such as oxygen and NADPH must be available for desaturase activity at the hardening temperature.

When plants of Phaseolus vulgaris are enclosed in polythene bags at 12 and 5°C it is hypothesized that the plants do not harden against chilling-injury at 5°C, 85 per cent RH, because the carbon dioxide concentration within the bag is rapidly reduced by photosynthetic fixation. This results in the cessation of photosynthesis and a reduced supply of the cofactors oxygen and NADPH available for desaturase activity (P. 180). Similarly, it is thought that Phaseolus vulgaris does not harden in the dark due to the ending of photosynthesis, resulting in a reduced supply of oxygen and NADPH, thereby limiting desaturase activity at a low level.

3. Why are extremely chill-sensitive species (e.g. Episcia reptans) incapable of rapid hardening?

According to the above hypothesis, Episcia reptans does not harden over 4 days at 15°C because the activity or quantity of desaturases is not increased or induced at the hardening temperature. Desaturase activity may, however, gradually increase over long periods of time at slowly decreasing temperature resulting in an increase in chill-resistance. Over evolutionary time spans the effect of constant high temperature on species such as Episcia reptans, which grow in tropical regions, may have resulted in the loss of the ability to increase desaturase activity over short periods at the hardening temperature, since little genotypic or phenotypic flexibility in relation to temperature may be needed for survival.

Alternatively, Episcia reptans may not harden over 4 days at 15°C due to an inadequate supply of cofactors for desaturase activity. Photosynthesis in Episcia reptans may be more sensitive to a decrease in temperature than in Phaseolus vulgaris and Gossypium hirsutum. Hence, in Episcia reptans, a reduced supply of oxygen and NADPH at the hardening temperature may restrict desaturase activity to a low level so that the degree of unsaturation of the phospholipids does not increase over the 4-day hardening period.

4. Is there a unifying role for the increases in unsaturation of the phospholipids during hardening against chilling and freezing-injury which affords protection against both types of injury?

Increases in unsaturation of the membrane-phospholipids on hardening chill-resistant species against freezing-injury may protect the leaf in the same kind of manner as the increases in unsaturation of the phospholipids on hardening chill-sensitive species against chilling-injury if the mechanisms of both types of injury are similar. Chilling-injury has been shown to be due to a phase change in the membrane lipids resulting in an increase in membrane permeability, perhaps due to the appearance of 'cracks' or 'holes' in the membrane. Similarly, freezing-injury is due to the formation of holes in the semi-permeable plasma membrane, leading to the loss of semi-permability, efflux of cell solution and consequent death. These holes may be produced by:-

1) Ice crystals penetrating the membrane during intracellular freezing.
2) Tension on the plasma membrane due to cell collapse during extracellular freezing. These holes may be

fixed irreversibly by intermolecular SS bonding of the membrane proteins (membrane-hole hypothesis, Levitt, 1972).

Therefore, it is suggested that the formation of these holes by intracellular freezing and freeze dehydration may be hastened by phase changes at sub-zero temperatures, especially in the 0 to -10°C range and in plants grown at high temperatures before freezing which may have a low degree of unsaturation of the phospholipids. Hardening may reduce the extent of freezing-injury by increasing the degree of unsaturation of the phospholipids thereby lowering the transition temperature. Little information is available on the behaviour of lipids and the occurrence of phase changes at sub-zero temperatures but Steim (1972) has detected a phase transition in Escherichia coli fatty-acids below 0°C. Obviously a phase change in the membrane lipids is not the sole cause of freezing-injury as some tissues can withstand -196°C without damage (Sakai, 1965), a temperature which must be well below the transition temperature of all membrane lipids.

1) Intracellular freezing

First, let us consider how an increase in unsaturation of the fatty-acids may prevent damage due to intracellular freezing. It is known that the cell membrane, due to its lipid nature, keeps extracellular ice from seeding the cell interior at temperatures above about -10°C, so that the cell remains unfrozen and supercooled. One explanation for the ability of the plasma membrane to prevent seeding by external ice above certain temperatures is that ice crystals small enough to

grow through aqueous channels, believed by some to exist in membranes, have melting points far below 0°C (Mazur, 1969). For example, an ice crystal of 30 angstroms in radius will melt above -10°C. If, however, a phase change occurred in the temperature range 0 to -10°C, cracks may appear in the membrane which would be large enough to permit the seeding of ice into the interior of the cell, producing severe injury. The gradual increase in membrane permeability during hardening against freezing-injury may also reduce the likelihood of intracellular freezing by facilitating the loss of water which could be frozen in the cell, thus allowing it to remain unfrozen and supercool. Increases in unsaturation and weight of phospholipids on hardening Hordeum vulgare and other chill-resistant species to sub-zero temperatures (Table 2) may therefore be important in the prevention of intracellular freezing.

2) Membrane-hole hypothesis

Secondly, according to the membrane-hole hypothesis (Levitt, 1972) holes may occur in the plasma membrane due to extracellular freezing producing cell contraction by dehydration and therefore a tension on the membrane. Covalent SS bonding between the protein layers may occur as a result of these holes, making the hole permanent, which leads to the efflux of cell contents on thawing and their death. A phase change in the membrane lipids at sub-zero temperatures would be expected to hasten injury by rapidly increasing membrane permeability and therefore cell contraction. In addition, a phase change at sub-zero temperatures may facilitate the formation of holes in the membrane due to the solidification of the lipids and a decrease in their flexibility. Openings in the membrane of this type may be prevented by an increase in unsaturation of the lipid layer during hardening, thus lowering the

temperature of the phase change and producing lipids with greater fluidity and expansibility at low temperatures which may permit the lipid to coalesce across the holes. Levitt (1972) has proposed that increases in the weights of phospholipid during hardening against freezing-injury are due to folding of the plasmallema which would reduce tension on the protoplast thus preventing covalent SS bonding between the protein layers of the membranes. However, no folding of the plasmallema has been detected by electron microscopy. Therefore, increases in unsaturation and weight of phospholipids on hardening against freezing-injury may have important roles in reducing the degree of damage due to the formation of holes by intracellular freezing and the membrane-hole hypothesis.

3) The effects of hardening on membrane structure

In addition to lowering the transition temperature of membrane lipids, increases in unsaturation of the fatty-acids and weight of phospholipids on hardening to chilling and freezing temperatures may also affect the structure of membranes. An understanding of how changes in lipid composition affect the structure of membranes is made difficult by the fact that almost as many membrane models have been proposed as there are investigators. Indeed, no single model seems capable of accounting satisfactorily for the properties of such widely differing systems as, for example, nerve cells and mitochondria. At present, thermodynamic considerations based on the theory that the steady state structure of membranes is one of lowest free energy indicate that the lipid globular protein mozaic model is the most satisfactory of current models for the gross molecular organisation of cellular membranes (Singer, 1971, see

Fig. 18). In spite of the differences in models of membrane structure, increases in unsaturation of the phospholipids on hardening can be expected to alter membrane structure by producing a decrease in ordered packing of the fatty-acid chains and perhaps alterations in their binding to the structural proteins. Whether hardening involves changes in structure from one membrane type to another is not known. Lucy (1968) has proposed that the adaptibility and versatility of biological membranes may result from the utilization of both globular micelles and bimolecular leaflet arrangements of lipid molecules in vivo. Differences in the flexibility of membranes may be due to differences in structure. For example, the flexibility of mitochondrial membranes isolated from chill-resistant fruits (Lyons et al., 1964) may be associated with micelles and rigidity of chill-sensitive mitochondria with the bimolecular leaflet.

Hardening against freezing-injury is not, however, solely due to changes in the lipid and fatty-acid composition of the membranes. Any mechanism opposing intracellular freezing or excessive dehydration and their effects (for a review see Mazur, 1969) should lead to a reduction in injury. Protection against freezing-injury can also be attributed to compounds such as sugars and protein hardiness factors (Heber, 1968), which may reduce the susceptibility of proteins to denaturation and aggregation. Nevertheless, the mode of interaction of sugars and protein hardiness factors with the membrane is still a matter of speculation. The incorporation of protein hardiness factors (Heber, 1968) into the membrane may also alter its molecular architecture.

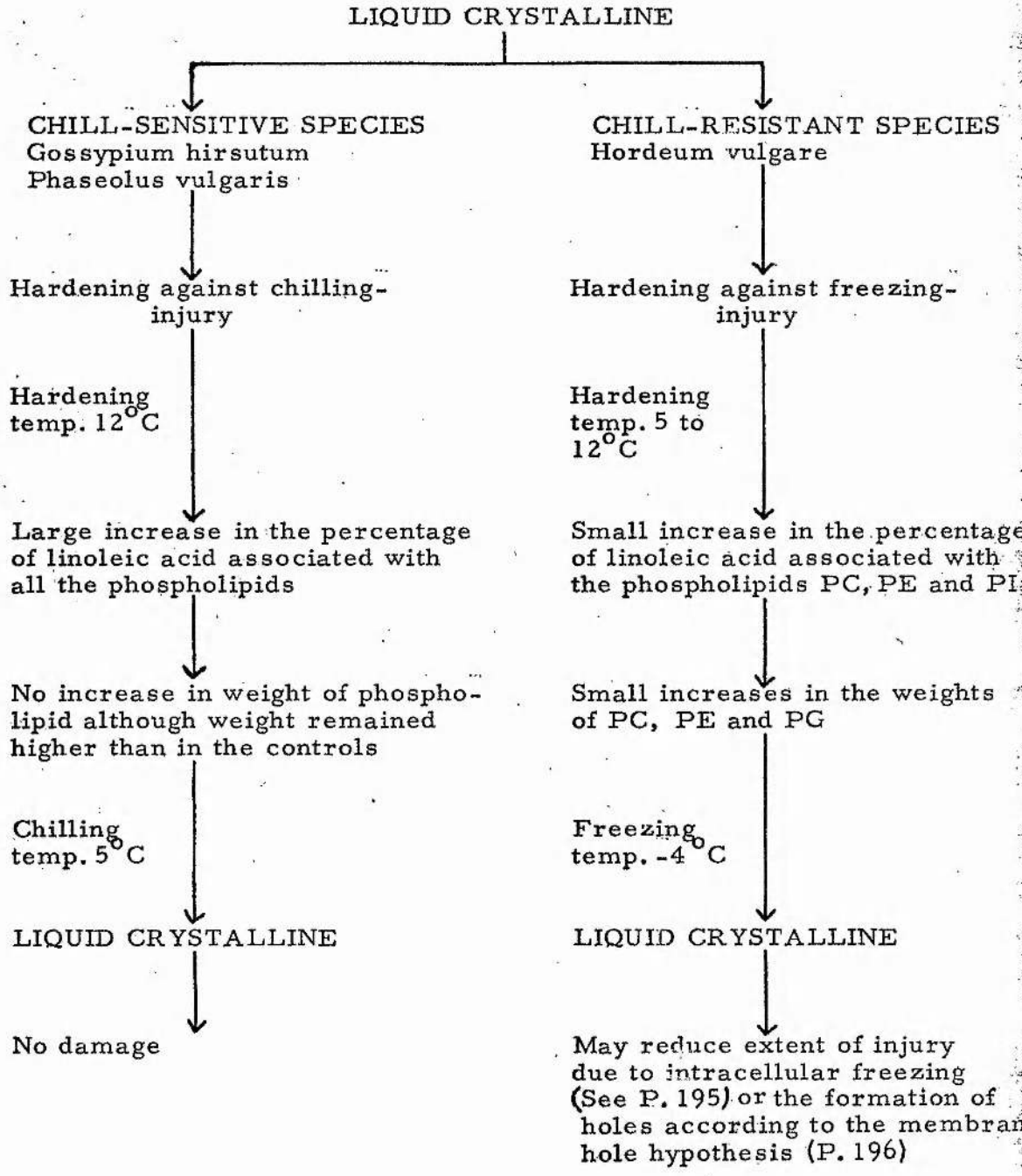
Therefore increases in unsaturation of the membrane phospholipids on hardening against chilling and freezing-injury may have a common

protective role in the prevention of both types of injury by lowering the transition temperature and producing alterations in membrane structure. A summary diagram of the changes in the degree of unsaturation of the phospholipids on hardening the chill-sensitive and chill-resistant species used in this study is shown in Fig. 19.

In conclusion, there now appears to be little doubt that the site of chilling and possibly freezing-injury lies in the cellular membranes. Phase changes may be contributory factors in the development of freezing-injury, especially in the 0 to -10°C range.

The resistance or sensitivity of a species to chilling temperatures appears to reside mainly in the degree of unsaturation of the membrane phospholipids. The ability of a chill-sensitive species to harden against chilling-injury is considered to be due to an increase in the activity or quantity of desaturases at the hardening temperature.

Fig.19. A comparison of the changes in the weight and fatty-acid composition of the phospholipids of chill-sensitive and resistant plant leaves during hardening and their role in the prevention of a phase change at chilling and sub-zero temperatures



FINAL SUMMARY

Chilling-injury has been investigated for many years in the fruits of tropical and sub-tropical species because lowered storage temperatures are generally an effective means of extending the market life of fruits and vegetables. However, the most striking visual effects and perhaps the most important ecological effects, in terms of range of survival of the species, may be considered to occur when the leaf is exposed to chilling temperatures. Therefore the ecological approach to chilling-injury is stressed in this investigation which used a total of 21 different species, 10 chill-sensitive and 11 chill-resistant species.

The extrapolation of the results obtained with fruits and vegetables to account for the behaviour of leaves in relation to chilling-injury can be misleading both physiologically and ecologically since many metabolic differences exist between growing leaves and mature fruits. For example, in contrast to fruits, the leaves of chill-sensitive species can be hardened to withstand chilling. Physiological studies of mitochondria isolated from fruits have led to general agreement that chill-resistance is associated with a high degree of unsaturation of the membrane fatty-acids thus preventing a phase change in the lipid layer of the membrane from a liquid-crystalline to a solid gel at chilling temperatures. Therefore, temperature acclimatization of the leaves afforded a unique opportunity, which was used in this thesis, to elucidate the biochemical mechanisms which enable plants to survive low temperature stress and thereby extend their geographical range.

Principal results

1. Chill-sensitive leaves can be divided into two categories based on their susceptibility to chilling-injury:-

   a) Extremely chill-sensitive species which are rapidly damaged on exposure to temperatures between 12 to 15°C (e.g. Episcia reptans) and which cannot be readily hardened against chilling-injury. Maintaining 100 per cent relative humidity during chilling at 5°C does not prevent injury to these species, although the speed at which injury occurs is reduced. Therefore, it is suggested that metabolic imbalances produced by the increase in E_a of membrane bound enzymes are the main pathway leading to injury in these species and that water loss may be of secondary importance.

   b) Chill-sensitive species which are damaged in the 0 to 10°C range (e.g. Phaseolus vulgaris) and which can be readily hardened against chilling-injury at 5°C, 85 per cent RH, by 4 days growth at 12°C, 95 per cent RH before chilling. Maintaining 100 per cent RH during chilling at 5°C prevents injury to these species. Therefore it is suggested that water loss is the main pathway leading to injury in these species and that metabolic imbalances produced by the increase in E_a of membrane bound enzymes are of secondary importance.

2. In plants grown at 25°C the degree of unsaturation of the total leaf fatty-acid of chill-resistant species was not higher than in the leaves

of chill-sensitive species. The chill-sensitivity of leaves grown at 25°C was, however, related to a low total percentage of linoleic and linolenic acid associated with each phospholipid, although the relationship was not precise in every phospholipid. Therefore, a comparison of the total percentage of linoleic and linolenic acid of all the leaf phospholipids combined showed the most marked relationship to the chill-sensitivity of the species. The degree of unsaturation of the glycolipids could not be related to the chill-sensitivity of the species and this is in agreement with the results of the analyses of the total leaf fatty-acid analyses of plants grown at 25°C.

3. Transfer from 25°C to 5°C, 85 per cent RH, produced a transitory increase in the ethanol content of chill-sensitive leaves. This is considered to be due to major imbalances in TCA and glycolytic activity as a result of a phase change in the membrane lipids of the mitochondria which increases the E_a of many TCA enzymes. Furthermore, chilling reduced the extent to which malonate inhibited oxygen uptake in chill-sensitive leaves, suggesting that TCA cycle activity was already adversely affected by the chilling treatment.

4. Transfer from 25°C to 5°C, 85 per cent RH, resulted in rapid decreases in the percentage of linolenic acid and total weight of fatty-acid in the total leaf extract of chill-sensitive species. In chill-resistant species the percentage of linolenic acid and weight of fatty-acid in the leaves remained unchanged on chilling at 5°C.

Rapid decreases in the weights of phospholipids and glycolipids in the leaves of chill-sensitive species on chilling suggested membrane breakdown due to enzymes such as phospholipase D and galactolipase.

5. Several chill-sensitive species e.g. Gossypium hirsutum, Phaseolus vulgaris and Cucumis sativus could be hardened against chilling-injury at 5°C, 85 per cent RH, by growing them for 2 to 4 days at 12°C, 95 per cent RH and 18-hour photoperiod of 71.8 W.M.$^{-2}$ before chilling. Young leaves of these species hardened more effectively than older leaves. Hardening leaves of Phaseolus vulgaris for 2 days at 12°C, 95 per cent RH, in the dark was not effective against subsequent chilling at 5°C, 85 per cent RH.

6. Hardening chill-sensitive plants produced no major increase in the degree of unsaturation or weight of fatty-acids in the total leaf and chloroplast fatty-acid extracts. Only a small increase in the percentage of linoleic acid of these extracts was detected.

7. Hardening resulted in increases of up to 12 per cent in the percentage of linoleic acid associated with all the leaf phospholipids of chill-sensitive Gossypium hirsutum and Phaseolus vulgaris. These increases in unsaturation were shown to be positively related to the increased tolerance of the plants to chilling by the fact that similar increases did not occur during the growth of chill-resistant Hordeum vulgare at 12°C and the ineffective attempts at hardening chill-sensitive Episcia reptans over 4 days at 15°C, the lowest temperature this species can withstand without injury.

8. Hardening against chilling-injury in the species investigated was shown to be related to an increase in unsaturation of the phospholipid fraction alone since no change occurred in the fatty-acid composition of the glycolipids on hardening. This accorded with earlier observations which showed no major increase in unsaturation of the total leaf and chloroplast fatty-acids on hardening. Therefore it is concluded that hardening prevents chilling-injury by increasing the degree of unsaturation of the phospholipids thereby lowering the transition temperature of the lipid layer of the membrane.

9. Leaves of Phaseolus vulgaris transferred from 25°C to 5°C, 100 per cent RH and 12°C, 100 per cent RH for 4 days, by enclosure in polythene bags, did not harden against subsequent chilling-injury at 5°C, 85 per cent RH. In agreement with this finding no increase in unsaturation of the phospholipids was detected over 4 days growth at 5°C, 100 per cent RH and 12°C, 100 per cent RH. It is suggested that plants grown at 5 and 12°C, 100 per cent RH, by enclosure in polythene bags do not harden against subsequent chilling-injury at 5°C, 85 per cent RH, because the concentration of carbon dioxide within the bag is rapidly reduced by photosynthetic fixation. This results in the cessation of photosynthesis and a reduced supply of the cofactors oxygen and NADPH available for desaturase activity. Similarly, it is thought that Phaseolus vulgaris does not harden in the dark at 12°C, 95 per cent RH, due to the ending of photosynthesis, resulting in a reduced supply of oxygen and NADPH, thereby limiting desaturase activity to a low level in the dark.

10. An increase in leaf age at 25°C was shown to increase the susceptibility of chill-sensitive plants to chilling-injury and chill-resistant plants to freezing-injury. This increase in susceptibility of older leaves to damage at either 5°C in the case of chill-sensitive plants, or -4°C in the case of chill-resistant plants, was related to a decrease in unsaturation and weight of phospholipids with increase in age at 25°C. The less effective hardening of older leaves of Gossypium hirsutum and Phaseolus vulgaris may also be related to this decrease in the degree of unsaturation of the phospholipids with increase in age. Therefore, leaves of young plants are better equipped to withstand low temperature stress at a critical stage in their establishment when many factors can determine the success or failure of a species in its habitat.

11. A comparison of hardening chill-sensitive species against chilling-injury and chill-resistant species against freezing-injury showed that changes in unsaturation and weight of lipids were similar. Therefore it is suggested that, in some instances, an increase in unsaturation and weight of phospholipid on hardening chill-resistant species against sub-zero temperatures may be important in reducing the severity of freezing-injury, either by preventing a phase change at sub-zero temperatures, or by producing a change in membrane structure.

12. In conclusion, the results reported in this investigation provide evidence that hardening chill-sensitive leaves

prevents chilling-injury by increasing the degree of unsaturation of the membrane phospholipids thereby lowering the temperature of the phase change in the lipid layer of the cellular membranes. This phase change does not occur on chilling chill-resistant plants but may occur at sub-zero temperatures and increase their susceptibility to freezing-injury.

BIBLIOGRAPHY

Adair, C. R. (1968). Testing rice seedlings for cold water tolerance. Crop Sci., 8, 264.

Allen, C. F., & Good, P. (1970). Acyl lipids in photosynthetic systems. Methods in Enzymology, 23, 523.

Amin, J. V. (1969). Some aspects of respiration and respiration inhibitors in low temperature effects of the cotton plant. Physiol. Plantarum, 22, 1184.

Apeland, J. (1966). Factors affecting the sensitivity of cucumbers to chilling temperatures. Bull. Int. Inst. Refrig., 46, Annexe 1, 325.

Appleman, C. O. & Smith, C. L. (1936). Effect of previous cold storage on the respiration of vegetables at higher temperatures. J. Agr. Res., 53, 557.

Artemova, E. K. & Zemlyanukhin, A. A. (1970). After-effect of temperature on fluoride and malonate inhibition of plant respiration. Fiziologiya Rastenii, 17, 962.

Barańska, J. & Wlodawer, P. (1969). Influence of temperature on the composition of fatty-acids and on lipogenesis in frog tissues. Comp. Biochem. Physiol., 28, 553.

Bierkander (1778) in "Responses of plants to environmental stresses" by Levitt, J. Academic press. P. 27.

Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37, 911.

Caldwell, R. S. & Vernberg, J. F. (1970). The influence of acclimation temperatures on the lipid composition of fish gill mitochondria. Comp. Biochem. Physiol., 34, 179.

Chapman, D., Kamat, V. B., De Gier, J. & Penkett, S. A. (1968). Nuclear magnetic resonance studies of erythrocyte membranes. J. Mol. Biol., 31, 101.

Christie, W. W. (1972). Quantitative esterification of lipids on thin-layer adsorbents. Analyst, 97, 221.

Christie, W. W., Noble, R. C. & Moore, J. H. (1970). Determination of lipid classes by a gas-chromatographic procedure. Analyst, 95, 940.

Christopherson, B. O. (1969). Reduction of linolenic acid hydroperoxide by a glutathione peroxidase. Biochim. Biophys. Acta. 176, 463.

Cobern, D., Hobbs, J. S., Lucas, R. A. & Mackenzie, D. J. (1966). Location of hydroperoxide groups in monohydroperoxides formed by chlorophyll-photosensitised oxidation of unsaturated esters. J. Chem. Soc. (C), 1897.

Cossins, E. A. & Beevers, H. (1963). Ethanol metabolism in plant tissues. Pl. Physiol., Lancaster, 38, 375.

Creencia, R. P. & Bramblage, W. J. (1971). Reversibility of chilling-injury to corn seedlings. Pl. Physiol., Baltimore, 47, 389.

Davis, D. L. & Finkner, V. C. (1972). Influence of temperature on sterol biosynthesis in Triticum aestivum. Pl. Physiol., Baltimore, 52, 324.

De Gier, J., Mandersloot, J. G. & Van Deenen, L. L. M. (1968). Lipid composition and permeability of liposomes. Biochim. Biophys. Acta., 150, 666.

De La Roche, I. A., Andrews, C. J., Pomeroy, M. K., Weinberger, P. & Kates, M. (1972). Lipid changes in winter wheat seedlings (Triticum aestivum) at temperatures inducing cold hardiness. Can. J. Bot., 50, 2401.

Dewey, M. M. & Barr, L. (1970). Some considerations about the structure of cellular membranes. Current topics in membranes and transport, 1, 1.

Eaks, I. L. & Morris, L. L. (1956). Respiration of cucumber fruits associated with physiological injury at chilling temperatures. Pl. Physiol., Lancaster, 31, 308.

Faris, J. A. (1926). Cold chlorosis of sugar cane. Phytopathology 16, 885.

Farkas, T. & Herodek, S. (1964). The effect of environmental temperature on the fatty-acid composition of crustacean plankton. J. Lipid Res., 5, 369.

Fraenkel, G. & Hopf, H. S. (1940). Temperature adaptation and the degree of saturation of the phosphatides. Biochem. J., 34, 1085.

Gerloff, E. D., Richardson, T. & Stahmann, M. A. (1966). Changes in fatty-acids of alfalfa roots during cold hardening. Pl. Physiol., Lancaster, 41, 1280.

Giaquinta, R. T. & Geiger, D. R. (1973). Mechanism of inhibition of translocation by localized chilling. Pl. Physiol., Baltimore, 51, 372.

Gibbs, M. (1971). Structure and function of chloroplasts. Springer-Verlag Berlin.

Grenier, G., Mazliak, P., Trémolières, A. & Willemot, C. (1973). Influence du froid sur la synthèse des acides gras dans les racines de deux variétiés de Luzerne, l'une très résistante et l'autre moins resistante au froid. Physiol. Vég., II(2), 253.

Guinn, G. (1971). Changes in sugars, starch, RNA, protein and lipid-soluble phosphate in leaves of cotton plants at low temperatures. Crop Sci., 11, 262.

Gurr, M.I. & James, A.T. (1971). In "Lipid biochemistry - an introduction." Chapman and Hall Ltd., London. P. 192.

Gunstone, F.D. (1967). An introduction to the chemistry and biochemistry of fatty-acids and their glycerides. Chapman and Hall.

Hamilton, S. & Hamilton, J. (1972). In "Topics in Lipid Chemistry". Vol. 3. Ed. F.D. Gunstone. Paul Elek (Scientific books) Ltd. P. 199.

Harris, P & James, A.T. (1969 a). The effect of low temperatures on fatty-acid biosynthesis in plants. Biochem. J., 112, 325.

Harris, P. & James, A.T. (1969 b). Effect of low temperature on fatty-acid biosynthesis in seeds. Biochim. Biophys. Acta., 187, 13.

Harris, R.V., James, A.T. & Harris, P. (1967). In "Biochemistry of chloroplasts". Vol. 2. Ed. T.W. Goodwin. Academic press. P. 250.

Harvey, R.B. (1922). Varietal differences in the resistance of cabbage and lettuce to low temperatures. Ecology, 3, 134.

Hatefi, Y. & Hanstein, W.G. (1970). Lipid oxidation in biological membranes. 1. Lipid oxidation in submitochondrial particles and microsomes induced by chaotropic agents. Archs. Biochem. Biophys., 138, 73.

Hawke, J.C. (1963). Studies on the properties of New Zealand butterfat. J. Dairy Res., 30, 67.

Heber, U. (1968). Freezing injury in relation to loss of enzyme activities and protection against freezing. Cryobiology, 5, 188.

Hilditch, T.P. (1956). The Chemical Constitution of Natural Fats. (Third Edition). Chapman and Hall.

Hitchcock, C. & Nichols, B. W. (1971). "Plant lipid biochemistry." Academic press, London and New York.

Holden, M. (1970). Lipoxidase activity of leaves. Phytochemistry, 9, 507.

Holton, W., Blecker, H. H. & Onore, M. (1964). Effect of growth temperature on the fatty-acid composition of a blue green alga. Phytochemistry, 3, 595.

Irving, L., Schmidt-Nielsen, K. & Abrahamsen, S. B. (1957). On the melting points of animal fats in cold climates. Physiol. Zool., 30, 93.

Jamieson, G. R. (1970). In "Topics in Lipid Chemistry." Vol. 1. Ed. F. D. Gunstone, London. Logos Press, P. 107.

Kates, M. (1970). Plant phospholipids and glycolipids. Adv. in Lipid Res., 8, 225.

Kislyuk, N. M. (1964). The effect of light on the injury to Cucumis sativus L leaves caused by severe cooling. Dokl. Acad. Nauk. SSSR, 158, 1434.

Kleinschmidt, M. G. & McMahon, V. A. (1970). Effect of growth temperature on the lipid composition of Cyanidium caldarium. Pl. Physiol., Baltimore, 46, 290.

Klopfenstein, W. E. & Shigley, J. W. (1967). Changes in fatty-acid composition of sulpholipid and phospholipids during maturation of alfalfa. J. Lipid Res., 8, 350.

Kodama, A. M. & Pace, N. (1963). Cold dependent changes in tissue fat composition. Fedn. Proc. Fedn. Socs. Exp. Biol., 22, 761.

Kolattukudy, P. E. (1965). Biosynthesis of wax in Brassica oleracea. Biochemistry, Easton, 4, 1844.

Kolattukudy, P. E. (1970). Plant waxes. Lipids, 5, 259.

Korovin, A. I. & Frolov, I. N. (1968). The effects of potassium and calcium on the resistance of buckwheat to low soil temperatures and freezing during the initiation of vegetation. Izv. Sib. Otd. Akad. Nauk. SSSR Ser. Biol. Med. Nauk., 3, 48.

Kuiper, P. J. C. (1970). Lipids in alfalfa leaves in relation to cold hardiness. Pl. Physiol. Baltimore, 45, 684.

Kuraishi, S., Arai, N., Ushijima, T. & Tazaki, T. (1968). Oxidised and reduced nicotinamide adenine dinucleotide phosphate levels of plants hardened and unhardened against chilling-injury. Pl. Physiol., Baltimore, 43, 238.

Ladbrooke, B. D. & Chapman, D. (1969). Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent studies. Chem. Phys. Lipids, 3, 304.

Levitt, J. (1969). Growth and survival of plants at extremes of temperature - a unified concept. Symp. Soc. Exp. Biol., 27, 485.

Levitt, J. (1972). Responses of plants to environmental stresses. Academic press, London and New York.

Lewis, D. A. (1956). Protoplasmic streaming in plants sensitive and insensitive to chilling temperatures. Science, 124, 75.

Lomagin, A. G. & Antropova, T. A. (1966). Photodynamic injury to heated leaves. Planta (Berl.), 68, 297.

Long, R. A., Hruska, F. E. & Gesser, H. D. (1971). Phase transitions in sphingomyelin thin films. Biochem. Biophys. Res. Commun., 45, 167.

Lucy, J. A. (1968). In "Biological membranes: physical fact and function." Ed. D. Chapman, London and New York.

Lyons, J. M. (1972). Phase transitions and control of cellular metabolism at low temperatures. Cryobiology, 9, 341.

Lyons, J. M. (1973). Chilling-injury in plants. Ann. Rev. Plant Physiol., 24, 445.

Lyons, J. M. & Asmundson, C. M. (1965). Solidification of unsaturated/saturated fatty-acid mixtures and its relationship to chilling-sensitivity in plants. J. Am. Oil Chem. Soc., 42, 1056.

Lyons, J. M. & Raison, J. K. (1970). A temperature-induced transition in mitochondrial oxidation: contrasts between cold and warm-blooded animals. Comp. Biochem. Physiol., 37, 405.

Lyons, J. M., Wheaton, T. A. & Pratt, H.K. (1964). Relationship between the physical nature of mitochondrial membranes and chilling-sensitivity in plants. Pl. Physiol. Lancaster, 39, 262.

Marr, A. G. & Ingraham, J. L. (1962). Effect of temperature on the composition of fatty-acids in Escherichia coli. J. Bact., 84, 1260.

Mazliak, P. (1973). Lipid metabolism in plants. Ann. Rev. Plant Physiol., 24, 287.

Mazur, P. (1969). Freezing-injury in plants. Ann. Rev. Plant Physiol., 20, 419.

Metcalfe, L. D., Schmitz, A. A. & Pelka, J. R. (1966). Rapid preparation of fatty-acid esters from lipids for gas-chromatographic analysis. Analyt. Chem., 38, 514.

Minchin, A. & Simon, E. W. (1973). Chilling-injury in cucumber leaves in relation to temperature. J. Expt. Bot., 24, 1231.

Molisch, H. (1896). Das Erfrieren von Pflanzen bei Temperaturen über dem Eispunkt. Sitzber. Kaiserlichen Akad. Wiss. Wien. Math. Naturwiss. Kl., 105, 1.

Molisch H. (1897). "Untersuchungen über das Erfrieren der Pflanzen." Fischer, Jena. P. 1 to 73.

Mooney, H. A. & Billings, W. D. (1961). Comparative physiological ecology of arctic and alpine populations of Oxyria digyna. Ecol. Monographs, 31, 1.

Murata, T. (1969). Physiological and biochemical studies of chilling-injury in bananas. Physiol. Plantarum, 22, 401.

Nayeeunisa (1966). Lipid metabolism in relation to thermal acclimation in earthworms. Ph. D. Thesis, Sri Venkateswara University, Tiripati, A. P., India.

Newman, D. W. (1962). Fatty-acids of bush bean leaf chloroplasts and proplastids. Biochem. Biophys. Res. Comm., 9, 179.

Nicholls, B. W. & James, A. T. (1964). The lipids of plant storage tissue. Fette Seifen Anstr-Mittel, 66, 1003.

Nichols, B. W., Stubbs, J. M. & James, A. T. (1967). In "Biochemistry of Chloroplasts." Ed. T. W. Goodwin. Vol. 2, P. 677, Academic press, London and New York.

Ongun, A., Thomson, W. W. & Mudd, J. B. (1968). Lipid composition of chloroplasts isolated by aqueous and nonaqueous techniques. J. Lipid. Res., 9, 409.

Pantastico, E. B., Grierson, W. & Soule, J. (1967). Chilling-injury in tropical fruits: 1. Bananas (Musa paradisiaca var. sapientum cv. Lacatan). Proc. Amer. Soc. Hort. Sci. Trop. Reg., 11, 82.

Pantastico, E. B., Soule, J. & Grierson, W. (1968). Chilling-injury in tropical and subtropical fruits. 2. Limes and grapefruit. Proc. Amer. Soc. Hort. Sci. Trop. Reg., 12, 171.

Patterson, G. W. (1970). Effect of culture temperature on the fatty-acid composition of Chlorella sorokina. Lipids, 5, 597.

Pearson, I. K. & Raper, H. S. (1927). The influence of temperature on the nature of fat formed by living organisms. Biochem. J., 21, 875.

Quarles, R. H. & Dawson, R. M. C. (1969). The distribution of phospholipase D in developing and mature plants. Biochem. J., 112, 787.

Raison, J. K. (1973). Temperature-induced phase changes in membrane lipids and their influence on metabolic regulation. Symp. Soc. Exp. Biol., 27, 485.

Rodniov, V. S., Nyuppieva, K. A. & Zakharova, L. S. (1973). Changes in the concentration of galacto- and phospholipids in potato leaves as a result of the influence of low temperatures. Fiziologiya Rastenii, 29, 525.

Roubal, W. T. & Tappel, A. L. (1966). Damage to proteins, enzymes and amino acids by peroxidising lipids. Archs. Biochem. Biophys., 113, 5.

Sachs, J. (1864). Ueber die obere Temperatur-Grenze der vegetation. Flora (Jena) 47, 5-12, 24-29, 33-39, 65-75.

Sakai, A. (1965). Survival of plant tissue at super low temperatures. 3. Relation between effective prefreezing temperatures and the degree of frost hardiness. Pl. Physiol., Lancaster, 40, 882.

Sastry, P. S. & Kates, M. (1964). Hydrolysis of monogalactosyl and digalactosyl diglycerides by specific enzymes in runner bean leaves. Biochem., 3, 1280.

Scholander, S. I. & Kanwisher, J. T. (1959). Latitudinal effect of respiration in northern plants. Pl. Physiol., Lancaster, 34, 574

Schwabe, W. W. (1957). In "Control of the plant environment." Ed. J. P. Hudson. Butterworths, London. P. 16.

Schwertner, H. A. & Biale, J. B. (1973). Lipid composition of plant mitochondria and chloroplasts. J. Lipid Res., 14, 235.

Sellschop, J. P. F. & Salmon, S. C. (1928). The influence of chilling above the freezing point on certain crop plants. J. Agr. Res., 37, 315.

Shneyour, A., Raison, J. K. & Smillie, R. M. (1973). The effect of temperature on the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants. Biochim. Biophys. Acta., 292, 152.

Siminovitch, D., Rheaume, B., Pomeroy, K. & Lepage, M. (1968). Phospholipid, protein and nucleic acid increases in protoplasm and membrane structures associated with the development of extreme freezing resistance in black locust tree cells. Cryobiology, 5, 202.

Singer, S. J. (1971). In "Structure and function of Biological Membranes." Ed. L. I. Rothfield. Academic press, London and New York.

Steim, J. M. (1972). Membrane transitions: Some aspects of structure and function. Fedn. European Biochem. Soc., Eighth meeting, Amsterdam. 28, 185.

Steim, J. M., Tourtellotte, M. E., Reinert, J. C., McElhaney, R. N. & Rader, R. L. (1969). Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. Proc. Natn. Acad. Sci. U. S. A., 63, 104.

Stewart, J. McD. & Guinn, G. (1969). Chilling-injury and changes in adenosine triphosphate of cotton seedlings. Pl. Physiol., Lancaster, 44, 605.

Taylor, A. O., Jepsen, N. M. & Christeller, J. T. (1972). Plants under climatic stress. III Low temperature, high light effects on photosynthetic products. Pl. Physiol., Baltimore, 49, 798.

Taylor, A.O. & Rowley, J.A. (1971). Plants under climatic stress. 1. Low temperature, high light effects on photosynthesis. Pl. Physiol., Baltimore, 47, 713.

Träuble, H. (1972). Phase transitions in lipids. Biomembranes, 3, 197.

Träuble, H. & Haynes, D.H. (1971). The volume change in lipid bilayer lamellae at the crystalline-liquid crystalline phase transition. Chem. Phys. Lipids, 7, 324.

Tsunoda, K., Fujimura, K., Nakahari, T., & Oyamado, Z. (1968). Studies on the testing method for cooling tolerance in rice plants. 1. An improved method by means of short turn treatment with cool and deep water. Jap. J. Breed., 8, 33.

Went, F.W. (1958). In "Physiological adaptation." Ed. C. L. Prosser. Amer. Physiol. Soc., Washington D.C. P. 126.

Wheaton, T.A. & Morris, L.L. (1967). Modification of chilling-sensitivity by temperature conditioning. Proc. Am. Soc. Hort. Sci., 91, 529.

Williams, M.A., Stancliffe, R.C., Packer, L. & Keith, A.D. (1972). Relation of unsaturated fatty-acid composition of rat liver mitochondria to oscillation period, spin label motion, permeability and oxidative phosphorylation. Biochim. Biophys. Acta., 267, 444.

Wilson, J.M. & Crawford, R.M.M. (1974a). Leaf fatty-acid content in relation to hardening and chilling-injury. J. Expt. Bot., 25, 121.

Wilson, J. M. & Crawford, R. M. M. (1974b). The acclimatization of plants to chilling temperatures in relation to the fatty-acid composition of leaf polar lipids. New Phytol., 73, 805.

Wright, M. (1971). Chilling-injury in cucumber seedlings. Ph. D. Thesis, Queens University, Belfast, Northern Ireland.

Wright, M. & Simon, E. W. (1973). Chilling-injury in cucumber leaves. J. Expt. Bot., 24, 400.

Yamaki, S. & Uritani, I. (1972). The mechanism of chilling-injury in sweet potato. 6. Changes in lipid components in the mitochondrial membrane during chilling storage. Pl. Cell Physiol., 13, 67.

Yoshida, S. & Sakai, A. (1973). Phospholipid changes associated with the cold-hardiness of cortical cells from poplar stem. Pl. Cell Physiol., 14, 353.

Zimmerman, D. C., Vick, B. A. & Borg, T. K. (1974). Intracellular distribution of hydroperoxide isomerase. Pl. Physiol., Baltimore, 53, 1.